BACTERIOLOGICAL ANALYTICAL MANUAL

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INTRODUCTION

The microbiological analytical work of the FDA is done largely in its field district laboratories. Uniformity of methods and of their application in these laboratories is essential. Accordingly, the Bacteriological Analytical Manual (BAM) has been prepared, primarily, in the interest of establishing and maintaining in FDA laboratories uniform analytical procedures for qualitative and quantitative determination of microorganisms in foods, drugs, cosmetics and certain other materials.

This second edition is a complete revision of the BAM published in 1965 and supersedes that collection of methods. It is of interest to note that the 1965 version was an outgrowth of bacteriological methods which were utilized internally as early as 1940. The aim then, as today, was to insure a uniform application of methods by microbiologists engaged in enforcing the provisions of the FD&C Act.

No claim is made that these methods are perfect, or even that they are superior to others that may be available. They are, however, those currently considered to be the most useful to the FDA. As in the past, revisions will be made as soon as methods or modification of methods better suited to our needs become evident.

In keeping with established FDA policy, the BAM also provides a mechanism for informing other Government agencies, industry, and others of the bacteriological methods commonly used in FDA district laboratories.

The BAM is the product primarily of those members of the Division of Microbiology most knowledgeable in the subject matter indicated by the respective methods. The Division staff is most appreciative of the many comments and suggestions for revisions that have been received from others; especially valuable were those from our district microbiologists. Also, we wish to express our sincere thanks to those individuals who so generously complied with our request to review certain sections of the Manual. As the need arises in the future, supplements and revisions of various sections of the Manual will be issued. In the interest of making such revisions more responsive to needs we shall welcome any constructive comments and suggestions toward improvement of this Manual.

Joseph C. Olson, Jr.
Director
Division of Microbiology

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1. CANNED FOODS

1.01 Equipment and materials

(a) incubators at 30°C, 35°C, 55°C

(b) pH meter

(c) microscope

(d) soap, water, brush

(e) towels (sterile and non-sterile)

(f) can opener

(g) sterile test tubes

(h) microscope slides and coverslips

(i) examination pans

(j) sterile serological pipets, cotton-plugged

(k) sterile non-tapered pipets, cotton-plugged (8 mm tubing)

(l) 2% iodine in 70% alcohol

(m) wax pencil or felt pen

(n) sterile petri dishes

(o) Bacti-disc cutter [1/]

(p) micro-leak detector

(q) non-foaming wetting agent

(r) micrometer

(s) sterile can punch

[1/] Purchased from Wilkens-Anderson Co., 4525 W. Division Street, Chicago, Ill. 60651 Cat. No. 4768.

1.02 Media

Bromcresol purple dextrose broth (BCP) (40.04); malt extract broth (40.24); acid broth (40.01); chopped liver broth (40.06) or cooked meat medium (40.07)[2/]; methylene blue stain (41.08)

1.03 Introduction

Can abnormality usually indicates an abnormal product. During progressive spoilage, cans may progress from normal to flipper to springer to soft swell to hard swell. Buckling or denting, closing while cool, overfilling, or prolonged storage may cause flippers or springers. Microbial spoilage or hydrogen from the reaction of acids with the metals of the cans may produce flippers, springers, or swells. Summer temperatures and high altitudes accentuate the degree of swelling. Not all microorganisms that grow in canned foods cause abnormal cans.

Spoilage within the can is caused by leakage or underprocessing. Leakage occurs from can defects, punctures, or rough handling. Contaminated cooling water sometimes leaks to the interior through pinholes or poor seams. The presence of a viable mixed flora of rods and cocci is indicative of leakage and should be confirmed by can examination. Underprocessing may occur from deliberate undercooking to preserve a "fresher" product, from faulty retort operations (including thermometers,

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[2/] Chopped liver broth is preferred.

gauges, and controls), from excessive contamination for which normally adequate processes are insufficient, or sometimes from accidental bypassing of the retort operation. When the can contains a spoiled product and no viable microorganisms, spoilage may have occurred before processing, or the microorganisms causing the spoilage may have died off during storage.

1.04 Can examination

Remove labels and transfer the subnumbers to the side of the can as an aid to correlate the findings with the codes.

Separate all cans by code numbers, if they are different, and examine them for any physical defects such as faulty closures, leaks, pinholes, buckling and dents. Classify each can condition as follows:[3/]

(a) Flipper. Only one end of the can is slightly bulged, and by finger pressure the end can be pressed back to remain flat. Or the end bulges when the can is struck sharply on a hard surface, and the bulged end can be pressed flat.

(b) Springer. One end of the can bulges, and when finger pressure is applied to press it flat, the opposite end bulges. Or both ends bulge and one end can be pressed flat.

3/ Cool to room temperature before classifying.

(c) Swell. Both ends bulge, but neither can be pressed flat. Bulged ends of soft swells yield slightly to finger pressure, but those of hard swells do not.

Set aside a reserve portion (702b) consisting of cans representing each condition noted and representative cans for analysis, e.g., if there are two springers, analyze one and place the other in the reserve sample. Record on the worksheet the condition of the cans to be analyzed.

1.05 Can incubation

Analyze all springers and swells immediately (1.07). Do not incubate. Place all normal cans and flippers in the incubator at 35°C. Examine them at frequent intervals for 14 days. When an abnormal can is found during this period, remove immediately and examine (1.07). After 14 days, remove the remaining normal cans and analyze a representative portion of these cans. (It is not necessary to analyze all normal cans.)

Incubation of cans at temperatures above 35°C need not be done unless there is reason to suspect that the product will be held at elevated temperatures in storage or in transit. Many canned foods contain a few thermophilic bacteria which do no harm under normal storage temperatures, but which will grow and spoil the product at 55°C (flat sour and thermophilic anaerobes).

1.06 Opening the can

Since the canned food method is essentially a test for the sterility of the product, the analysis should be performed in a clean atmosphere (preferably a bacteriological hood) to preclude any possibility of laboratory contamination.

Scrub the entire container with a brush, using warm water and soap on the uncoded end of the can. Rinse and dry with a clean towel. Chill hard swells in the refrigerator before opening them.

Flame the uncoded end by holding over a bunsen burner or similar device. Do not flame hard swells; use 2% iodine in 70% alcohol and wipe away with a sterile towel.

Hydrogen swells sometimes occur in acid canned foods from the action of the acid in the food on the metal in the container. If a test for hydrogen is to be made, hold a sterile test tube near the site of the opening and make a small puncture in the flamed surface, catching some of the escaping gas in the tube. Immediately flip the mouth of the tube to a bunsen burner flame. An explosion indicates the presence of hydrogen in the head space. (Do not flame the hole. Serious explosions have occurred from this technique.) Enlarge the opening in the can, using the Bacti-disc cutter to permit removal of the sample.

Remove portions of the contents from the center of the can, well removed from the site of the opening, and place in appropriate media (1.07). Sample all products, liquid or solid, using sterile wide-mouth pipets or sterile glass tubing if a larger size opening is desired. Do not use forceps, spoons, or other instruments to sample solid products, since this may introduce outside contamination into the sample. Solid products usually have enough liquid so that a sample may be obtained. Sterile water can be introduced, mixed, and removed to media if the product is too solid. Never pipet by mouth unless the pipet is plugged.

Transfer portions from the contents of representative cans to sterile stoppered test tubes and refrigerate at ca 5°C. Use these for repeat examination, if questionable results are obtained after incubation, and for possible toxicity tests.

1.07 Cultural and direct microscopic tests

[Use either (a) or (b)]

(a) Non-acid foods[4/] (pH above 4.5). From each container inoculate 4 tubes of chopped liver broth or cooked meat medium

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4/ Non-acid foods are meats, fish, milk, and most vegetables. They generally spoil from spore-forming anaerobes or facultative anaerobes, which may be mesophilic or thermophilic.

previously heated to 100°C and cooled to room temperature or below.[5/] Also inoculate 4 tubes of bromcresol purple dextrose broth (BCP). Incubate as follows:

Medium	No. of Tubes	Temp, °C	Time of Incubation, hr	To Test For
Chopped liver (cooked meat)	2	35	96	Putrefactive anaerobes
Chopped liver (cooked meat)	2	55	72[6/]	Thermophilic anaerobes
BCP	2	55	48[6/]	Flat sour thermophiles
BCP	2	35	96	Leakage types

(b) Acid foods[7/] (pH below 4.5). Test abnormal cans for hydrogen (1.06). From each container inoculate, with ca 1 ml of product, 4 tubes of acid broth and 2 tubes of malt extract

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5/ Chopped liver broth or cooked meat medium is heated and cooled to remove oxygen inhibitory to the growth of anaerobes.

6/ Also examine at 24 and 48 hr.

7/ Acid foods are tomatoes, fruits and fruit juices, fruit drinks, pickles, jams, jellies, and catsup. Syrups and concentrates may generally be treated as acid foods. Acid foods may spoil from yeasts, molds, or lactobacilli, as well as acid-tolerant sporulating thermophilic or mesophilic anaerobes.

broth. Incubate as follows:

Medium	No. of Tubes	Temp. °C	Time of Incubation, hr	To Test For
Acid broth	2	55	48	Flat sour thermophiles
Acid broth	2	30	96	Yeasts, molds, lactobacilli
Malt extract broth	2	30	96	Yeasts, molds, lactobacilli

During inoculation (1.07) (a) or (b) note whether the food itself introduces turbidity into the medium. After incubation, do not record such turbidity as positive unless it proves to be growth after transfer to a fresh medium, or by direct microscopic examination.[8/]

1.08 Direct microscopic examination

Prepare a smear from the contents of each can after subculturing. Dry, fix, and stain with methylene blue stain; then examine under the microscope. If the product is oily, drop xylene over a warm, fixed slide, rinse, and stain. If the product washes off the slide during preparation, examine as a wet mount or hanging drop,

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[8/] Be certain that the microorganisms seen under the microscope are viable. Dead organisms may sometimes be carried over from the inoculum or the chopped liver.

or prepare a suspension of the test material in a drop of chopped liver broth before drying. Check the liver broth microscopically before use to be sure it is free of microorganisms.

1.09 Examination of contents

After culturing:

(a) Determine pH of each subsample with a pH meter.

(b) Cut out most of the end of the can, using the Bacti-disc cutter, and pour contents into examination pans. Examine for odor, color, consistency and texture; examine the can lining for blackening, detinning, and pitting. Do not taste the product.

1.10 Seam evaluation

(a) Micro-leak detection [see Bee and Denny, Ref. (8)]. Remove the uncoded end with a Bacti-disc cutter adjusted to cut out most of the can end, leaving 1/4 inch around the outer edge. Empty and wash the container; add a nonfoaming wetting agent[9/] to a depth of ca one inch.

9/ "Seam Test Type U", Winton Products Co., Inc., Box 3332, Charlotte, North Carolina 28203.

Place the plastic Plexiglass plate with Plexiglass tubing and the wetted rubber gasket for the particular container size on the open end of the container. Increase the vacuum slowly until the gauge indicates a vacuum normally obtained with the product in question. Swirl the water in the container to dissipate the small bubbles occurring from the application of the vacuum.

Tilt the container slowly to immerse all seam surfaces. Depending on the size of the leak, bubbles will appear as a chain originating from one location or as a large, growing bubble. Release the vacuum by first closing the main vacuum petcock, and then opening the intake petcock.

(b) Tear-down examination. Use appropriate methods such as those described in "Evaluating a Double Seam" by W. R. Grace & Co., Cambridge, Mass.

2. DETECTION OF PREFORMED CLOSTRIDIUM BOTULINUM TYPES A, B OR E TOXIN IN FOOD SAMPLES

DO NOT START THIS ANALYSIS UNTIL YOU HAVE BEEN PROTECTED BY SUITABLE TOXOIDS.

2.01 Equipment and materials

(a) 14 mice for each sample (32 mice for positives)

(b) types A, B, and E antisera[1/]

(c) Difco trypsin (1:250 activity), 10% solution in distilled water

(d) inoculating syringes (sterile)

(e) Millipore or fritted glass UF filter

(f) 37°C water bath

(g) centrifuge

(h) 70% ethyl alcohol

(i) 1 N HCl

(j) sterile mortar and pestle; sterile sand or mechanical blender

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1/ Obtain antisera directly from National Communicable Disease Center, Atlanta, Ga. 30322 or Division of Microbiology, Food and Drug Administration, Washington, D. C. 20204. If lyophilized, reconstitute with sterile 50% glycerol in water, in accordance with label instructions. Dilute sera 1:10 with sterile saline for mouse injection.

2.02 Reagents

Saline, sterile, 0.85% (41.10); gel-phosphate diluent (41.06)

2.03 Preparation of food extract

(a) If canned foods are to be tested, scrub the uncoded can end with a brush, using warm water and soap; rinse, and dry with a towel. Number each can on the side, as an aid to correlate findings with code. (Chill hard swells in the refrigerator before opening them.)

Open the can as directed in BAM 1.06. Special care should be taken when opening swollen cans to avoid creating an aerosol.

Record the condition of the can and its content.

(b) Liquid food samples or can washings can be tested for the presence of toxin without further preparation.

(c) Suspected food remnants can be prepared in the same manner as canned solid foods.

Homogenize the sample (either a portion of the can contents or food remnant) with an equal weight of sterile gel-phosphate diluent, using a mechanical blender. With some foods or remnants it may be necessary to grind the sample in a mortar.

Add an equal weight of sterile gel-phosphate diluent and a small amount of sterile sand (depending on the volume to be ground) and grind with a sterile pestle until a homogeneous suspension is obtained.

2.04 Preparation of filtrates

Centrifuge a portion of the homogenate in the cold at high speed for 30 minutes. Filter through a sterile Millipore or fritted glass filter (not Seitz) to obtain the clear, fat-free liquid. Check the pH. If above 6.5, adjust to 6.0-6.2 with 1 N HCl. Freeze for overnight storage, if necessary.

2.05 Trypsinization and heat treatment

To 2 ml of the filtrate adjusted to pH 6.0-6.2 (2.03) add 0.2 ml of the 10% solution of trypsin. Incubate in a water bath at 37°C for 45 minutes. Cool. Do not store trypsinized extract overnight.

Heat a portion of the sterile filtrate for 10 minutes at 100°C.

2.06 Screening inoculations

Dilute both trypsinized (2.04) and nontrypsinized (2.03) filtrates 1:2, 1:10, and 1:100 in gel-phosphate diluent.

Inject 0.5 ml of each dilution intraperitoneally into 2 mice (total of 12 mice). In addition, inject 2 mice intraperitoneally with 0.5 ml of the undiluted heated filtrate. Record their condition at frequent intervals during 48 hours. Deaths occurring with either the trypsinized or nontrypsinized unheated filtrate are presumptive evidence of toxin, and should be confirmed with protected mice (2.07).

2.07 Confirmation with protected mice

Dilute a new portion of the nontrypsinized (2.03) or trypsinized (2.04) filtrate (whichever showed the highest titer) to 1:2, 1:10, and 1:100 in gel-phosphate diluent. (Do not store trypsinized material overnight.)

Inject each of 8 mice intraperitoneally with 0.5 ml of a 1:10 saline dilution of type A antiserum, each of 8 mice with 0.5 ml of a 1:10 saline dilution of type B antiserum, and each of 8 mice with 0.5 ml of a 1:10 saline dilution of type E antiserum. Leave 8 mice without this protection. After 30 minutes, inject 0.5 ml of each dilution of the filtrate into each of 2 mice previously protected with either type A, type B or type E antisera and into 2 unprotected mice.

Record their condition at intervals up to 48 hours. If the unprotected mice die and the group of mice protected with either type A, type B, or type E antisera live, the presence of either type A, type B, or type E toxin is indicated. If all these protected mice die, send the filtrate on dry ice to Division of Microbiology for further tests.

3. DETECTION OF CLOSTRIDIUM BOTULINUM TYPES A, B AND E IN FOOD SAMPLES[1/]

DO NOT START THIS ANALYSIS UNTIL YOU HAVE BEEN PROTECTED BY SUITABLE TOXOIDS.

3.01 Equipment and materials

(a) 16 (18-24 g) mice for each sample (32 mice for positives)

(b) types A, B, and E antisera[2/]

(c) inoculating syringes (sterile)

(d) sterile inoculating instruments

(e) 35°C incubator

(f) 26°C incubator

(g) centrifuge

(h) sterile centrifuge tubes

(i) Millipore or fritted glass UF filters

[1/] If a product is suspected of being contaminated with botulinum toxin or organisms (i.e., food poisoning), then both methods (BAM 2 and 3) should be used. If the product is being routinely analyzed for botulinum contamination (i.e., no suspected contamination), only the BAM 3 method should be used unless toxin is found; then use the BAM 2 method also.

[2/] Obtain antisera directly from National Communicable Disease Center, Atlanta, Ga. 30322 or Division of Microbiology, Food and Drug Administration, Washington, D. C. 20204. If lyophilized, reconstitute with sterile 50% glycerol in water, in accordance with label instructions. Dilute sera 1:10 with sterile saline for mouse injection.

3.02 Media and reagents

TPGYT (40.34); chopped liver broth (40.06); gel-phosphate diluent (41.06); saline, sterile, 0.85% (41.10).

3.03 Cultures

Inoculate 3-4 g portions of the sample into each of 3 tubes of freshly boiled and cooled chopped liver broth. At the same time, inoculate 3-4 g portions of the same sample into each of 3 tubes of TPGYT broth. Incubate the inoculated chopped liver tubes at 35°C for 5 days. Incubate the inoculated TPGYT broth tubes at 26°C for 5 days.

3.04 Preparation of filtrates

Following incubation, combine aliquots of the chopped liver broth cultures and separately combine aliquots of the TPGYT cultures. Centrifuge each of the combined aliquots in the cold at high speed for 30 minutes. Filter each supernatant through a sterile Millipore or fritted glass filter (not Seitz) to obtain a clear, fat-free liquid. Freeze for overnight storage, if necessary.

3.05 Heat treatment

Heat a portion of each sterile culture filtrate for 10 minutes at 100°C.

3.06 Screening inoculations

Dilute each filtrate 1:5, 1:10 and 1:100 in gel-phosphate diluent. Inject 0.5 ml of each dilution of each filtrate intraperitoneally into 2 mice (total of 12 mice). In addition, inject 2 mice intraperitoneally with 0.5 ml of each undiluted heated filtrate. Record their condition at frequent intervals during 48 hours. Deaths occurring with either or both of the unheated filtrates is presumptive evidence of toxin, and should be confirmed with passively protected mice (3.07).

3.07 Confirmation with protected mice

Determine which culture filtrate showed the highest titer in mice. Dilute a new portion of this filtrate 1:5, 1:10, and 1:100 in gel-phosphate diluent.

Inject each of 8 mice intraperitoneally with 0.5 ml of a 1:10 saline dilution of type A antiserum, each of 8 mice with 0.5 ml of a 1:10 saline dilution of type B antiserum, and each of 8 mice with 0.5 ml of a 1:10 saline dilution of type E antiserum. Leave 8 mice without this protection. After 30 minutes, inject 0.5 ml of each dilution of that filtrate which showed the highest titer into each of 2 mice previously protected with either type A, type B or type E antisera and into 2 unprotected mice.

Record their condition at intervals up to 48 hours. If the unprotected mice die and the group of mice protected with either type A, type B, or type E antisera live, the presence of either type A, type B, or type E toxin is indicated. If all these protected mice die, send the filtrate on dry ice to Division of Microbiology for further tests.

4. PROCEDURE TO DETERMINE THE PRESENCE OF CLOSTRIDIUM BOTULINUM TYPE E IN SMOKED FISH

DO NOT START THIS ANALYSIS UNTIL YOU HAVE BEEN PROTECTED BY SUITABLE TOXOIDS.

4.01 Equipment and materials

(a) 8 (16-24 g) mice for each subsample (16-24 mice for positives)

(b) types A, B, and E antisera[1/]

(c) inoculating syringes (sterile)

(d) 26°C incubator

(e) Millipore or fritted glass UF filters

(f) centrifuge

(g) water-tight plastic bags

(h) sealing device for sealing plastic bags

4.02 Media and reagents

TPGYT (40.34); gel-phosphate diluent (41.06); saline, sterile, 0.85% (41.10).

[1/] Obtain antisera directly from National Communicable Disease Center, Atlanta, Ga. 30322 or Division of Microbiology, Food and Drug Administration, Washington, D. C. 20204. If lyophilized, reconstitute with sterile 50% glycerol in water, in accordance with label instructions. Dilute antisera 1:10 with sterile saline for mouse injection.

4.03 Incubation

Place each smoked fish subsample (this may consist of 1 or more fish depending on size, and may be either vacuum packed or bulk smoked fish) in a strong, water-tight, plastic bag. Add ca 100 ml of TPGYT broth to the subsample. Squeeze the bag to exclude as much air as possible and have the subsample essentially submerged in the culture medium. Seal the bag with a hot iron or some other sealing device. Incubate at 26°C for 5 days. Precautions should be taken during the incubation period, since the bag may swell and split.

4.04 Cultures

Centrifuge a portion of the TPGYT broth after incubation, and filter through a sterile Millipore or fritted glass filter (not Seitz) to obtain the clear, fat-free liquid.[2/] Freeze for overnight storage, if necessary.

4.05 Screening inoculations

Dilute the sterile culture filtrates (4.04) 1:5, 1:10, 1:100 and 1:1000 in gel-phosphate diluent. Inject 0.5 ml of each dilution intraperitoneally into each of 2 mice (total of 8 mice). Record their condition at frequent intervals during 48 hours. If no deaths occur, no further tests are indicated.

[2/] Chill tubes before centrifuging or use refrigerated centrifuge.

Deaths are presumptive evidence of toxin, and should be confirmed with protected mice (4.06). Freeze for overnight storage, if necessary.

4.06 Confirmation with protected mice

Dilute a new portion of the sterile culture filtrates (4.04) 1:5, 1:10, 1:100, and 1:1000 in gel-phosphate diluent.

Inject each of 8 mice intraperitoneally with 0.5 ml of a 1:10 saline dilution of type E antiserum. Leave 8 mice without this protection. After 30 minutes, inject 0.5 ml of each dilution into each of 2 mice previously protected with antiserum and into each of 2 mice not so protected.

Record their condition at intervals up to 48 hours. If the unprotected mice die and the protected inoculated mice live, the presence of type E toxin is indicated. If all the protected mice die, repeat serological neutralization tests with mice protected with Clostridium botulinum types A and B antisera.

Inject each of 8 mice intraperitoneally with 0.5 ml of a 1:10 saline dilution of type A antiserum and each of 8 mice with 0.5 ml of a 1:10 saline dilution of type B antiserum. Leave 8 mice without this protection. After 30 minutes, inject 0.5

ml of each dilution of sterile culture filtrate into each of 2 mice previously protected with types A and B antiserum and into 2 mice not so protected.

Record their condition at intervals up to 48 hours. If the unprotected mice die and the group of mice protected with either type A or type B antisera live, the presence of either type A or type B toxin is indicated. If all these protected mice die, send the filtrate on dry ice to Division of Microbiology for further tests.

Isolate and identify cultures from samples containing toxin of type E (BAM 5).

5. ISOLATION OF CLOSTRIDIUM BOTULINUM TYPE E IN SMOKED FISH

DO NOT START THIS ANALYSIS UNTIL YOU HAVE BEEN PROTECTED BY SUITABLE TOXOIDS. USE THE SAFETY HOOD FOR HANDLING CULTURES.

5.01 Equipment and materials

(a) sterile inoculating instrument

(b) 35°C incubator

(c) 26°C incubator

(d) sterile pipets

(e) 16 to 40 (18-24 g) mice per positive subsample

(f) anaerobic atmosphere (N_2) or other anaerobic apparatus to create an anaerobic atmosphere

(g) absolute ethyl alcohol

(h) sterile syringes

(i) sterile test tubes

(j) Millipore or fritted glass UF filter

(k) type E antiserum (see footnote under BAM 4.01)

5.02 Media

TPGYT (40.34); anaerobic egg agar (40.02); gel-phosphate dilu[illegible] (41.06); saline, sterile, 0.85% (41.10).

5.03 Isolation of pure cultures (To be done only if toxin is confirmed.)

To 1 or 2 ml of the toxic culture (BAM 4.03) (especially the sediment) add an equal volume of absolute ethyl alcohol in a sterile test tube. After 1 hour at room temperature, streak a large loopful of the mixture to anaerobic egg agar, and pipet 0.1 ml into TPGYT broth.

Incubate the plates anaerobically at 35°C for 48 hours and the inoculated TPGYT broth aerobically at 26°C. Look for typical colonies[1/] of type E on the plates after 48 hours of incubation.

5.04 Toxicity of TPGYT cultures

If plates contain apparently pure cultures, let the TPGYT broth inoculated at the time of plate streaking (5.03) incubate for 3 days at 26°C; then filter a portion of the culture through a Millipore or fritted glass filter (not Seitz). Freeze for overnight storage, if necessary.

1/ Colonies of type E are white, flat, and irregular, 1-2 mm in diameter, surrounded by a zone of yellow precipitate ca 2 to 4 mm in diameter. The precipitation zone surrounding the colony is iridescent (pearly-layer) to oblique light. The iridescent precipitation zone is characteristic of several other species of the genus Clostridium, so be careful in the interpretation of results. Streak stock cultures to gain experience.

5.05 Toxicity tests

Dilute the sterile culture filtrates (5.04) 1:5, 1:10, 1:100, and 1:1000 in gel-phosphate diluent. Inject each of 2 mice intraperitoneally with 0.5 ml of each dilution. Record their condition at frequent intervals up to 48 hours. If animals die, confirm the nature of the toxin (5.06).

5.06 Confirmation of type E toxin in cultures

Dilute a new portion of sterile culture filtrate (5.04) 1:5, 1:10, 1:100, and 1:1000 in gel-phosphate diluent.

Inject each of 8 mice intraperitoneally with 0.5 ml of a 1:10 saline dilution of type E antiserum. Leave 8 mice without this protection. After 30 minutes, inject 0.5 ml of each dilution into each of 2 mice previously protected with antiserum and into each of 2 mice not so protected.

Record their condition at intervals up to 48 hours, If the unprotected mice die and the protected mice live, the presence of type E toxin is indicated.

5.07 Picking colonies

If plates appear contaminated, discard the TPGYT inoculated in parallel with the streaking, and pick at least 10 typical

isolated colonies[2/] to TPGYT broth. Incubate 3 days at 26°C. Prepare culture filtrates, dilute 1:100 in gel-phosphate diluent, and test for toxin, using protected and unprotected mice (5.04-5.06).

[2/] Many colonies will fail to be toxigenic. Pick at least 10 typical colonies to locate a toxin-producing colony.

6. ISOLATION OF CLOSTRIDIUM PERFRINGENS (WELCHII)[1/]

6.01 Sample collection and storage

Sample the entire portion of food if possible (whole roast, chicken, etc). Transport and examine the sample promptly without freezing. Freeze only if the sample cannot be analyzed within 1 or 2 days.[2/]

6.02 Equipment and material

(a) anaerobic atmosphere (N_2) or other apparatus to create anaerobic atmosphere

(b) sterile mortar and pestle and sterile sand, or a two-speed Waring Blendor

(c) 26°C incubator

(d) 46°C water bath

1/ *Clostridium perfringens* food poisoning may occur when meat or poultry is cooked and held without adequate refrigeration prior to serving. The oxygen level is sufficiently reduced during cooking to permit growth of the clostridia.

Illness occurs 8-15 hours after ingestion, with intense intestinal cramps, gas and diarrhea; nausea and vomiting are rare. Victims usually recover rapidly but serious illness may occur occasionally as a result of dehydration and circulatory failure.

2/ Freezing may destroy 99% or more of the *Clostridium perfringens* vegetative cells; even refrigeration without freezing may kill 90%.

6.03 Media and reagents

Chopped liver broth (40.06) or cooked meat medium (40.07)[3/]; liver veal egg yolk (LVEY) agar (40.22); indole nitrite medium (40.18) or motility-nitrate medium (40.25); Ellner's sporulation broth (40.11); sulfite-polymyxin-sulfadiazine (SPS) agar (40.32) or tryptone-sulfite-neomycin (TSN) agar (40.36); fluid thioglycollate medium (40.12); iron milk medium (40.19); peptone water diluent (41.11); Gram stain reagents; Reagent I (41.12); Reagent II (41.13)

6.04 Direct microscopic examination

Prepare a smear, Gram stain, and examine for Gram-positive rods.

6.05 Cultural tests

If on direct examination typical cells were few or if the sample was grossly contaminated with aerobic microorganisms, inoculate into fluid thioglycollate and incubate at 46°C (6.06). If there were numerous typical Gram-positive rods and few contaminants, inoculate into chopped liver broth (6.07) or cooked meat medium. Do plate counts for sulfite-reducing clostridia on all samples (6.08).

[3/] Chopped liver broth is preferred.

6.06 Fluid thioglycollate medium

Inoculate 2 or 3 g portions taken at random[4/] into several tubes of fluid thioglycollate medium. Incubate in a water bath at 46°C for 4-8 hours. Subculture positive tubes in chopped liver broth or cooked meat medium (6.07).

6.07 Chopped liver broth or cooked meat medium

Inoculate 2 or 3 grams of the sample into each of several tubes or chopped liver broth or cooked meat medium previously boiled and cooled.[5/] Incubate aerobically for 24-48 hours. Observe cultures for profuse gas production and examine for characteristic Gram-positive rods. Streak positive tubes to LVEY plates. Incubate plates anaerobically for 24-48 hours at 35°C and examine for colonies producing lecithinase zones.[6/] Confirm as Clostridium perfringens (6.09).

6.08 Plate count of viable clostridia

If the sample is a solid, macerate a portion of it with peptone water in a mortar with sterile sand or blend[7/] with peptone

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[4/] The organisms may be unevenly distributed in the sample. Include both surface and deep portions.

[5/] Heat all liquid media in boiling water or flowing steam for 10 minutes to expel oxygen just prior to use.

[6/] Opalescent zone 2-5 mm in diameter surrounding the colony.

[7/] Blend carefully; excessive exposure to oxygen may kill the clostridia.

water for 1 minute in a Waring Blendor at low speed. Make serial dilutions in peptone water with gentle mixing. Prepare pour plates in SPS agar or TSN agar and, after the agar has solidified, overlay with 5 ml of the same medium. Incubate plates anaerobically at 35°C for 18-24 hours. Count black (sulfite-reducing) colonies. Pick several colonies for confirmation tests (6.09).

6.09 Confirmation as Clostridium perfringens

Pick typical colonies from LVEY (6.07) and SPS (6.08) to chopped liver broth, cooked meat medium, or fluid thioglycollate medium. Incubate 18-24 hours at 35°C. When a pure culture has been obtained, streak 2 plates of LVEY and incubate one anaerobically, the other aerobically. Report which plate supports growth. Inoculate iron milk and incubate 24 hours at 35°C. Inoculate duplicate tubes of indole nitrite or motility-nitrate medium by expelling a column of inoculum into the chilled semisolid medium while withdrawing a 1 ml pipet.

Incubate indole nitrite or motility-nitrate medium 18-24 hours at 26°C. Examine for motility[8/]; then add 5 drops of reagents

8/ Cl. perfringens is non-motile. Growth should occur only along the line of inoculum and not diffuse throughout the entire medium.

I and II to tubes showing heavy growth. A red or pink color indicates reduction of nitrate to nitrite. Include a negative control. In iron milk, look for vigorous gas production and early coagulation (stormy fermentation). Report as Clostridium perfringens nonmotile, spore-forming[9/] obligate anaerobes that reduce nitrate to nitrite and produce stormy fermentation in iron milk.

[9/] Cl. perfringens does not sporulate readily. To produce spores, use a sporulation broth such as Ellner's medium. Inoculate heavily with a young culture and incubate 24 hours in a water bath at 37°C.

7. ANALYSIS OF CRABMEAT AND LOBSTERMEAT

7.01 Sample collection, transport, and storage

Sample the entire container, if possible, or if the containers are too large, aseptically sample ca 200 g into a sterile container for each subsample.

It is preferable to examine a minimum of 10 subsamples of each kind of meat or 10 subsamples of the type of meat which would best show the conditions in the plant. As soon as the sample is collected, place the subsamples in an ice chest in wet ice. Transport the sample in wet ice and store the sample in the refrigerator at 3-5°C, and start the analysis within 48 hours after collection. Freezing is undesirable because indicator organisms may be destroyed.

7.02 Preparation of sample

Clean the top and edge of the can and wipe with 70% alcohol. If the can is hermetically sealed, open as directed in BAM 1.06.

7.03 Examination

Determine aerobic plate count, coliform group, *Escherichia coli*, and coagulase-positive Staphylococcus according to JAOAC 49 (1), 246-250 (1966), and JAOAC 51 (2), 505 (1968).

8. DRUGS AND DEVICES: STERILITY

8.01 Introduction

The FD&C Act requires that if a drug is described in an official compendium, determinations as to purity shall be made in accordance with the tests set forth in such compendium. This means that USP or NF products must be tested for sterility in accordance with the latest USP or NF. Nothing in what follows should be construed to alter the instructions detailed in these compendia.

Sterility tests are highly exacting and should be conducted only by personnel who have had expert training and experience in rigid aseptic techniques.

The USP describes in detail the equipment and conditions necessary for a proper aseptic filling operation. Although it does not describe the environment required for sterility testing we must be certain that the microorganisms we find in sterility testing come from the product and not from the environment. For this reason, we must be certain that our laboratory testing facilities at least equal the USP specifications for aseptic filling areas. Read Remington's Pharmaceutical Sciences (Martin, 1965). You must be prepared to defend your procedure in court.

8.02 Equipment and materials

(a) the sterility test area should conform to the requirements for aseptic filling areas (USP).

(b) 2, 5, 20, or 30 ml syringes, Luer-Lok, with 1-1/2 inch #20 needles. (Use smaller or larger needles for some products.)

(c) sterile files, forceps, scissors, scalpels, etc., as required.

(d) an effective disinfectant.

(e) sterile pipets of appropriate sizes.

(f) surgical gown, face mask, and elbow length rubber gloves.

8.03 Media and reagents

Fluid thioglycollate medium (40.12); fluid thioglycollate medium, azolectin-Tween modification (40.13) for products containing quaternary ammonium compounds; fluid Sabouraud medium (see USP). Dispense media to size tubes listed in USP. Butterfield's phosphate diluent (41.03).

8.04 Preparation of media and equipment

Prepare and sterilize your own media and equipment, or supervise the work closely so that you are absolutely sure of its sterility.

(a) Sterilize pipets and other equipment with dry heat at 170°C for 3 hours or overnight at 160°C. This equipment

should be sterilized the day before use and set aside in a clean, protected place apart from the equipment used for other analysis, to insure against any possibility of recontamination.

(b) Steam sterilize media according to USP directions. Identify sterilizer recorder charts with sample number, type of media, initials, and date, and submit the chart with the sample work-sheets. Incubate entire batches of media before use according to USP directions.

(c) Use laminar flow hoods located in a separate room or a clean area which can be easily cleaned and disinfected. Disinfect interior of hood with an effective chemical disinfectant (isopropyl alcohol diluted with water to a concentration of 60-70% is one of the best disinfectants for Gram-negative and Gram-positive organisms, and acid-fast bacilli). Turn the fan on for at least 1 hour (preferably overnight) before analysis is started.

8.05 Bacteriostasis

Using the procedure described in USP, determine the bacteriostasis of the product in fluid thioglycollate medium, using Cl. perfringens, B. subtilis, and E. coli, and the fungistatic activity of the product in Sabouraud medium, using a 1:1000

dilution of a 24-48 hour culture of C. albicans. Once the bacteriostasis is determined, keep a record for future reference. The record should include brand name, product, formulation, suspected bacteriostat, dilution found effective, inactivating agent found effective, test organisms used, results, and difficulties encountered. USP requires the use of an inactivating agent, if available, and permits dilution beyond bacteriostatic level only as a second choice. Use azolectin-Tween thioglycollate medium for products containing quaternary ammonium compounds.

8.06 Inoculation

Always wear elbow length rubber gloves and sanitize them. Wear sterilized surgical gowns and masks.

(a) Liquids, suspensions, surgical dressings, etc.: Proceed as directed in USP.

(b) Devices (drapes, blankets, transfusion sets, catheters, syringes, and other bulky items): Some of these items are so bulky that suitable containers would be difficult to find. For items that can be sampled by removing pieces from the unit, cut approximately 1 inch square portions from at least 6-10 different locations and place each into suitable quantities of medium.

For devices in which the sterility of the fluid path is critical, testing should be done by passing a volume of sterile test medium through the fluid path sufficient enough to thoroughly wash all sides of each unit.

Syringes or other small devices which are individually packaged and sterilized should be immersed in the test medium if possible. Be sure the medium gets into the fluid path of the syringe and needle, if attached.

8.07 Controls

Using fluid thioglycollate medium, test a representative number of pipets, syringes, or other equipment being used. Expose agar plates and/or open tubes of sterile media around the working area in the hood.

8.08 Incubation

Incubate inoculated tubes at 30-32°C (thioglycollate) or at 22-25°C (Sabouraud) for times specified in USP (note that incubation times vary with the product).

If the product is a device that was sterilized by irradiation, incubate tubes for 18-20 days before discarding them as negative.

(a) Examine incubating tubes each workday. When a positive is found, confirm growth as indicated below, and arrange for a check analysis at an approved facility on additional units without waiting until the entire incubation period is over. This may save several days.

(b) Transfer to a solid medium, incubate, and Gram stain; report staining and morphological characteristics.[1/] Transfer a loopful from positive thioglycollate tubes to additional thioglycollate medium and incubate to confirm that cloudiness was caused by microbial growth.

(c) When an ophthalmic solution is found non-sterile, serially dilute a portion of the reserve subsample in Butterfield's phosphate diluent and determine aerobic plate count, incubating plates at 30-32°C for 3 days. If, during the examination of an ophthalmic solution, there is any reason to suspect that contaminating organisms are pathogenic (injury report, or other reasons) identify the organisms, but only to the point where they are eliminated as possible pathogens. Except when pathogens are strongly suspected to be present, do not attempt to identify contaminants.

Consult the latest USP for further details.

[1/] Bacteria from thioglycollate will give improper Gram stain and frequently fail to sporulate, so be careful in reporting the staining characteristics and presence or absence of spores.

9. FUNGICIDES IN GRAIN[1/]

9.01 Introduction

The method will detect Captan, tetramethylthiuram disulfide, ethyl mercuryphosphate, ethyl mercury-p-toluene sulfonanilide, methyl mercuridicyandiamide, ethyl mercurychloride, Vancide-89, chlorophenyl mercury sulfate, phenylmercuric acetate, phenylmercuric ammonium acetate, and phenylmercuric urea. It does not detect hexachlorobenzene and pentachloronitrobenzene. The method has been successfully used for wheat, corn, barley, and beans. The method is nonspecific, and incapable of identifying or measuring the quantity of the fungicide on the grain.

9.02 Equipment and materials

(a) large heat-resistant glass tray or baking dish, preferably 16 x 24 inches

(b) N-ethylmercury-p-toluene sulfonanilide (Ceresan M), 4 µg/ml in 70% alcohol; store in tightly stoppered bottle

(c) glass plates or other suitable cover for tray

(d) Sarcina lutea, ATCC 9341

(e) vacuum seed sampler, or tweezers and paper template

(f) petri dishes

(g) 35°C incubator

(h) sterile 125 ml Erlenmeyer flask containing glass beads

[1/] Molinas (16).

9.03 Medium

Penassay seed agar (40.30) in slants and in 100 ml quantities. Saline, sterile, 0.85% (41.10).

9.04 Preparation of suspension

Inoculate the entire surface of 2 or more fresh slants with Sarcina lutea, ATCC 9341. Incubate 24 ± 2 hours at 35°C. Wash the growth from each slant aseptically with 5 ml sterile saline into a sterile 125 ml Erlenmeyer flask containing glass beads. Shake gently and store in the refrigerator. Replace with a fresh preparation each month.

To determine the optimum inoculum add 0.1 ml, 0.2 ml, and 0.5 ml of the Sarcina lutea suspension to each of three 100 ml portions of melted penassay seed agar cooled to 45°C. Pour inoculated agar into sterile petri dishes fo form a layer ca 2 mm deep. When the agar is gelled, but not yet completely hardened, place a circle of filter paper which has been dipped into Ceresan M solution into the surface, and invert. Incubate at 35°C overnight. Choose for an inoculum the level that gives the largest and most distinct zone of inhibition.

9.05 Screening grain for fungicides

Melt penassay seed agar and cool to 45°C. Add the appropriate inoculum of the Sarcina lutea suspension. Shake gently and

pour into petri dishes or trays to form a layer 2 mm deep. Allow to harden slightly; while agar is still soft, distribute kernels evenly over the surface at ca 15 mm intervals.[2/] Replace the cover and incubate at 35°C overnight. Clear zones (absence of growth) around kernels indicate the presence of a fungicide.

Absence of a fungicide is indicated by growth of the test organism up to the kernel of grain. The sensitivity of the test will vary with the fungicide, the depth of agar, and other factors such as the diffusibility of the fungicide and the quantity present in the portion of the kernel in contact with the agar.

Report number of kernels tested and number positive.

[2/] Place a template under the tray and place kernels with tweezers; or use a vacuum or manual seed counting head.

10. ISOLATION OF SALMONELLA

10.01 Introduction

Isolating Salmonella from various food products often requires methods different from those used in clinical or public health laboratories. During the processing or storage of food products, the organisms have sometimes been subjected to heat, desiccation, preservatives, freezing, changes in osmotic pressures, and changes in pH. These may weaken the vitality of the organisms. Salmonellae usually comprise a relatively small proportion of the total microbial flora of food products.

In general, pre-enrich all foods that have received a dry heat treatment (desiccated, dehydrated, or powdered) in a non-inhibitory broth to initiate growth of salmonellae. Such foods usually have a reduced microbial population so that salmonellae, if present, are less likely to be overgrown by other organisms. Raw foods and finished products that have been grossly contaminated after processing should be examined by direct enrichment in selective broths to prevent overgrowth by other microorganisms.

It is extremely important to adjust the pH as recommended in order to obtain the best results.

Somatic O antisera and the polyvalent flagellar H antiserum do not possess antibodies necessary to react with all of the 1,000+ Salmonella types now accepted in the Kauffmann-White schema.

Negative serological results will occur with some Salmonella types when tested with these antisera. The identification of such cultures must be resolved by biochemical tests and further serological analysis.

10.02 Equipment and materials

(a) blender and sterile blender containers with lids

(b) sterile wide-mouth screwcap pint jars

(c) sterile glass rods

(d) sterile spoons

(e) sterile pipets

(f) sterile petri dishes

(g) weighing balance

(h) 35°C hot air incubator

(i) 50°C water bath

10.03 Media and reagents

Distilled water, 200 ml (40.09); distilled water, 1000 ml (40.10); nonfat dry milk solution (40.27); nutrient agar

(40.28); nutrient broth (40.29); crystal violet solution (41.04); brilliant green solution (41.02); Tergitol Anionic 7 (41.14).

10.04 Dried eggs and dried egg products, prepared powdered mixes (cake, cookie, doughnut, biscuit and bread), infant formula

NOTE: THIS IS THE REFERENCE METHOD.

Examine, using method for "Detection and Identification of Salmonella in Egg Products", JAOAC 50 (1), 231-239 (1967), as revised in JAOAC 51 (2), 505-506 (1968).

10.05 Dyes and coloring substances

(a) Products with a pH of 6.0 or above (10% aqueous suspension): Use Reference Method (10.06)

(b) Examine laked aluminum dyes and dyes with a pH below 6.0 by aseptically weighing 25 g portion into sterile wide-mouth screw cap pint jar. Add 225 ml of brilliant green tetrathionate broth, and mix well. Loosen jar cap approximately 1/4 turn and incubate 24 ± 2 hours at 35°C. Streak selective agar plates according to second paragraph, section 37.X19, and continue examination thru section 37.X28 of Reference Method (10.04).

10.06 Liquid eggs or frozen egg products (whole egg, egg white or egg yolks)

Thaw frozen product as rapidly as possible to prevent increase in number of microorganisms present and at temperature low enough to prevent destruction of salmonellae (not greater than 45°C for not longer than 15 minutes). Frequent rotary shaking aids in thawing and increases uniformity of product.

(a) For pasteurized liquid eggs or frozen egg products: Use Reference Method (10.04).

(b) For nonpasteurized liquid eggs or frozen egg products: Aseptically weigh duplicate 25 g portions of product into separate sterile wide-mouth screwcap pint jars. Add 225 ml of selenite cystine broth to one jar and add 225 ml of brilliant green tetrathionate broth to the other jar. Shake well. Loosen jar cap approximately 1/4 turn and incubate 24 ± 2 hours at 35°C. Streak selective agar plates according to second paragraph, section 37.X19, and continue examination thru section 37.X28 of Reference Method (10.04).

10.07 Dried yeast (inactive)

Aseptically weigh 50 g into sterile wide-mouth screwcap pint jar. Add 200 ml of sterile distilled water, and mix well. If pH is below 6.6, adjust to 6.8 ± 0.2 with sterile 1 N NaOH.

Loosen jar cap approximately 1/4 turn and incubate 24 ± 2 hours at 35°C. Continue examination according to sections 37.X19-37.X28 of Reference Method (10.04).

10.08 Coconut

Aseptically weigh 25 g into a sterile wide-mouth screwcap pint jar. Add 225 ml of sterile lactose broth, and shake well. Adjust pH to 6.8 ± 0.2 with sterile 1 N NaOH or HCl. Add 2.2 ml of Tergitol Anionic 7 (sodium heptadecyl sulfate). Mix well. Loosen jar cap ca 1/4 turn and incubate 24 ± 2 hours at 35°C. Continue examination according to sections 37.X19-37.X28 of Reference Method (10.04).

10.09 Egg-containing foods (egg noodles, egg rolls, etc.)

Aseptically weigh 25 g into a sterile blending container. Add 225 ml of sterile lactose broth, and blend for 2 minutes. Aseptically transfer blended mixture to a sterile wide-mouth screwcap pint jar, and shake well. If pH is below 6.6, adjust to 6.8 ± 0.2 with sterile 1 N NaOH. Loosen jar cap ca 1/4 turn and incubate 24 ± 2 hours at 35°C. Continue examination according to sections 37.X19-37.X28 of Reference Method (10.04).

10.10 Dried milk

Aseptically weigh 100 g of dried milk into a sterile 2 L flask

(or wide-mouth screwcap 2 quart jar). Add 1000 ml of sterile distilled water. If pH is below 6.6, adjust to 6.8 ± 0.2 with sterile 1 N NaOH or HCl. Add 4 ml of 1% aqueous crystal violet or 2 ml of 1% aqueous brilliant green, and mix well. Incubate 24 ± 2 hours at 35°C. Continue examination according to sections 37.X19-37.X28 of Reference Method (10.04).

10.11 Meat, animal feed, animal substances, glandular products or fish meal

(a) For heated, processed or dried product: Aseptically weigh 25 g of product into a sterile blending container. Add 225 ml of sterile lactose broth, and blend for 2 minutes. Aseptically transfer the blended mixture to a sterile, wide-mouth, screwcap pint jar. (If product is a powder, ground or comminuted, blending may be omitted; aseptically weigh 25 g into a sterile jar, and add 225 ml of sterile lactose broth and mix well.) If pH is below 6.6, adjust to 6.8 ± 0.2 with sterile 1 N NaOH. Add 2.2 ml of Tergitol Anionic 7 (sodium heptadecyl sulfate) if product contains a large quantity of fat. Incubate 24 ± 2 hours at 35°C.

Shake incubated product-lactose broth mixture gently, transfer 0.5 ml to 10 ml of selenite cystine broth, and transfer

an additional 0.5 ml to 10 ml of brilliant green tetrathionate broth. Incubate 24 ± 2 hours at 35°C. Streak incubated selenite cystine broth and brilliant green tetrathionate enrichment broth onto selective agar plates according to second paragraph of section 37.X19 and continue examination through section 37.X28 of Reference Method (10.04).

(b) For raw or highly contaminated meat, animal feed, animal substances or glandular products: Aseptically weigh duplicate 25 g portions into separate sterile blending containers. Add 225 ml of selenite cystine broth to one container and 225 ml of brilliant green tetrathionate broth to the other. Blend each container for 2 minutes. Aseptically transfer blended mixture to separate sterile, wide-mouth, screwcap pint jar. (If product is powder, ground or comminuted, blending may be omitted; aseptically weigh duplicate 25 g portions into separate sterile, wide-mouth, screwcap pint jars. Add 225 ml of selenite cystine broth to one jar and 225 ml of brilliant green tetrathionate broth to the other and mix well.) Incubate 24 ± 2 hours at 35°C. Streak incubated selenite cystine broth and brilliant green tetrathionate enrichment broth onto selective agar plates according to second paragraph of section 37.X19, and continue examination thru section 37.X28 of Reference Method (10.04).

10.12 Candy, candy coating

Aseptically weigh 100 g of candy into a sterile blending container. Add approximately 300 ml from a liter of autoclaved reconstituted nonfat dry milk solution (100 g of nonfat dry milk + 1000 ml of distilled water, autoclaved 20 minutes at 121°C). Blend for 2 minutes. Aseptically transfer the blended mixture back to the remaining portion (ca 700 ml) of nonfat dry milk solution. If pH is below 6.6, adjust pH to 6.8 ± 0.2 with sterile 1 N NaOH. Add 4 ml of 1% aqueous crystal violet or 2 ml of 1% aqueous brilliant green and mix well. Incubate 24 ± 2 hours at 35°C. Continue examination according to sections 37.X19-37.X28 of Reference Method (10.04).

10.13 Frosting mixes, topping mix (Mixes with large quantity of sugar)

Aseptically weigh 25 g into a sterile, wide-mouth, screwcap pint jar. Add 225 ml of sterile nutrient broth. If pH is below 6.6, adjust to 6.8 ± 0.2 with sterile 1 N NaOH. Loosen jar cap ca 1/4 turn and incubate 24 ± 2 hours at 35°C. Continue examination according to sections 37.X19-37.X28 of Reference Method (10.04).

11. ISOLATION OF SHIGELLA

11.01 Introduction

Shigellosis is a serious problem in the U. S. Currently the incidence of this disease is well in excess of 7000 cases per year. Food-borne outbreaks usually result from the consumption of contaminated foodstuffs such as potato salads served by food handlers who were previously ill with the disease. The extensive carrier state that is characteristic of this organism causes secondary attacks due to hand-to-mouth contacts. The attack rate per outbreak of shigellosis is 2-1/2 times that of salmonellosis.

Shigella in many respects duplicates the behavior of Salmonella as an infective and contaminating agent. Thus it is likely that the frequency of input of these organisms into foodstuffs is the same as or even greater than that of salmonellae. It is possible that interstate foods may be a vector in the dissemination of this organism.

11.02 Suspect foods

Suspect foods would be those types requiring hand processing or minimal heating before consumption. They could be products

derived from animal sources, or products freshly delivered to consumer outlets. They are more likely to be products whose pH falls in the range of 5.5-7.5. In general, foods showing a high incidence of coliforms, E. coli, and salmonellae are suspect for shigellae.

11.03 Preview of the isolation and identification of Shigella

Briefly the method calls for inoculation of the foodstuff into enrichment broths consisting of GN and selenite cystine broths. This is followed by streaking onto XLD, DC, and EMB isolation agars. Suspicious colonies from these are inoculated onto TSI agar slants. If the growth from this produces an acid butt and an alkaline slant, the organism is nonmotile and urease-negative; it is inoculated into KCN, malonate, and IMViC media. The organism should be MR-positive and indole positive or negative, but negative in the remaining tests. It is then inoculated into one group of sugar media in which it is inert, producing neither acid nor gas. These consist of lactose, sucrose, salicin, inositol, and adonitol broths. This is followed by a final group of sugar media in which no gas is produced, but acid may or may not be produced. This last group consists of glucose, maltose, arabinose, xylose, and mannitol broths. The organism conforming to these criteria is considered to be a presumptive member of the Shigella group.

Any isolate with presumptive membership in the Shigella group is tested against Shigella somatic polyvalent antisera containing poly groups A, B, C, D, and A-D. Regardless of a positive or negative serological response, the organism should be further tested for confirmation of the genus finding, as well as for species and serotype identification.

11.04 Equipment and materials

(a) sterile wide-mouth screwcap jars, pints

(b) 35°C incubator

(c) sterile glass rods

(d) petri plates

(e) blender and sterile jars

(f) Shigella somatic antisera poly groups A, B, C, D, and A-D

11.05 Media and reagents

Brain heart infusion (BHI) (40.03); carbohydrate fermentation broth (40.05); desoxycholate citrate agar (DC) (40.08); Gram-negative broth (GN) (40.14); Koser's citrate medium (40.15); MR-VP medium (40.16); tryptone broth (40.17); KCN broth (40.20); Levine's eosine methylene blue agar (EMB) (40.21); malonate broth (40.23); motility test medium (40.26); nutrient agar (40.28); nutrient broth (40.29); selenite cystine broth (40.31);

triple sugar iron agar (TSI) (40.33); urea broth (40.37); xylose lysine deoxycholate agar (XLD) (40.39); alpha-naphthol solution (41.01); Butterfield's buffered phosphate diluent (41.03); Kovac's reagent (41.07); methyl red solution (41.09); saline, 0.85% solution (41.10); Voges-Proskauer reagent (V-P) (41.15).

11.06 Enrichment (parallel culture)

(a) Weigh 10 g of the sample into 20 ml of GN broth contained in a screw cap jar and stir with a sterile glass rod until the product is completely dispersed. Add additional GN broth equivalent to a total of 240 ml. Close the jar and shake to suspend the sample. Loosen the cap to permit the escape of gas. Incubate overnight (18 hours or less) at 35°C and streak onto selective agars as soon as possible the next morning. Do not discard the jars but incubate another 24 hours. Streak once again on selective agars.

(b) Selenite cystine broth. Repeat the process, weighing 25 g of the sample into a final total of 225 ml of selenite cystine broth. Incubate for 18 hours or less at 35°C. Streak onto selective agars, regardless of visible growth. Incubate an additional 24 hours and streak selective agars again.

11.07 Selective agars (parallel culture)

XLD, DC, EMB selective agars are streaked by means of a 3 mm loop from GN and selenite cystine enrichment broths. The selective plates are incubated for 24 ± 2 hours at 35°C. Select representative, well-isolated, suspicious[1/] colonies and subculture onto TSI slants. If no growth is evident on the selective plates, incubate another 24 hours and subculture any suspicious colonies onto TSI.

11.08 TSI

Slant-streak and butt-stab the fished colonies from selective agars onto TSI agar slants. Incubate for 24 ± 2 hours[2/] at 35°C.

On TSI the reaction of Shigella is an alkaline (red) slant and an acid (yellow) butt, with negative gas and negative H_2S production. Such a reaction is suspicious for Shigella. Suspicious TSI cultures are subcultured into urea broth, motility test medium, nutrient agar, and nutrient broth.

[1/] Shigella will appear on all selective media as gray or slate gray, entire, glistening, viscid colonies by reflected light, varying in size according to the inhibitory medium. On XLD agar they will appear to be rose colored, surrounded by a rosy halo, when viewed by transmitted light.

[2/] Readings of TSI beyond 24 hours are not valid. After this the entire TSI tube turns alkaline in reaction. If for any reason a longer time interval elapses before the tube can be read (weekend), a fresh tube of TSI should be inoculated.

11.09 Preliminary tests, inoculated from TSI

(a) Urea broth. Inoculate a urea broth tube from TSI. Incubate 24 ± 2 hours at 35°C. Cultures producing a red color (alkaline reaction) are urease positive. Shigella is urease negative.

(b) Motility test medium. Inoculate a tube of motility test medium from TSI by stabbing the top of the column of medium to a depth of approximately 5 mm. Incubate for 1-2 days at 37°C. A circular growth from the line of stab constitutes a positive test for motility. Shigella is nonmotile.

(c) Nutrient agar. Inoculate a tube of nutrient agar from TSI. Use growth from this medium as a source of inoculum and for test of purity[3/] of culture. Perform a Gram stain and examine microscopically. Shigella is a Gram-negative rod.

(d) Nutrient broth. Inoculate a tube of nutrient broth from TSI. Use growth from this medium as a source of inoculum and for a check on motility, should any doubtful reaction occur in the motility test medium.

3/ The purity of a suspected culture must be assured before further work is done on it. If a culture is suspected of being impure, make a light suspension in 0.85% NaCl. Streak the suspension and work it out well on BHI agar plates by means of a needle. Do not use inhibitory media, because it is desirable that the contaminating organism express its presence by means of a visible colony, so that it can be avoided. The Shigella organism is a Gram-negative rod, 1-3 μ long by 0.4-0.6 μ wide, non-capsulated, non-sporeforming and occurring singly.

11.10 Further biochemical tests

If the suspicious organism as tested under 11.09 is Gram-negative, nonmotile, and urease negative, inoculate the following test media: KCN broth, malonate broth, tryptone broth, MR-VP broth, and Koser's citrate broth as described below.

(a) KCN broth. Inoculate KCN broth with a 3 mm loopful of a 24 hour nutrient broth culture and incubate for 48 ± 2 hours at 35°C. Positive results are indicated by turbidity in the tube. Shigella is negative.

(b) Malonate broth. Inoculate malonate broth with a 3 mm loopful of a 24 hour nutrient broth culture and incubate for 48 ± 2 hours at 35°C. A positive reaction is indicated by a change in the color of the indicator from green to dark blue. Shigella is negative.

(c) Indole test. Perform the indole test by inoculating a tryptone broth tube from the nutrient agar slant. Incubate the culture for 48 ± 2 hours at 35°C. Test by adding 0.5 ml of Kovac's reagent and shaking the tube gently. A positive test is indicated by the formation of a deep red color. Shigella is either positive or negative.

(d) Methyl red and V-P test. Inoculate a tube of MR-VP medium by means of a needle from the nutrient agar culture. Incubate

at 35°C for 48 ± 2 hours. To 1 ml of this culture add 0.2 ml of 40% KOH. Shake well; add 0.6 ml of 5% alpha-naphthol. Add a few crystals of creatine and shake well. A red color forming in 2-4 hours constitutes a positive V-P test. Shigella is V-P negative.

Incubate the remainder of MR-VP medium for an additional 48 ± 2 hours. Add a few drops of methyl red solution to the culture. A distinct red color constitutes a positive reaction. A yellow color is a negative reaction. Shigella is MR-positive.

(e) Citrate utilization. Inoculate a tube of Koser's citrate medium by means of a needle from the nutrient agar culture, effecting the barest transfer of cells to the citrate medium by inoculating the side of the tube just below the surface of the medium. Incubate at 35°C for 4 or 5 days. A positive test consists of visible, turbid growth in the medium. Shigella is negative.

11.11 Fermentation reactions in carbohydrate media in which Shigella is inert

Inoculate lactose, sucrose, salicin, inositol, and adonitol broths from the nutrient agar culture. Incubate at 35°C for

4 or 5 days and examine daily for acid and gas production. *Shigella* produces neither acid nor gas in these sugars.

11.12 Fermentation reactions in carbohydrate media in which *Shigella* produces no gas

Inoculate glucose, maltose, arabinose, xylose, and mannitol broths from the nutrient agar culture. Incubate for 4 or 5 days and examine daily for acid and gas production. *Shigella* produces no gas.[4/] *Shigella* may or may not produce acid from these carbohydrate media.

11.13 Recapitulation of Shigella group characteristics

Gram stain - Gram-negative rod

motility - nonmotile

urease - negative

H_2S - negative

KCN broth - no growth

malonate - no growth

indole - negative or positive

methyl red - positive

Voges-Proskauer - negative

citrate utilization - negative

lactose - negative acid

[4/] *S. flexneri 6* can produce gas from glucose and mannitol.

sucrose - negative acid

salicin - negative acid

inositol - negative acid

adonitol - negative acid

glucose - negative gas; ± acid

maltose - negative gas; ± acid

arabinose - negative gas; ± acid

xylose - negative gas; ± acid

mannitol - negative gas; ± acid

Any microorganism that produces H_2S in TSI and is a urease positive, motile form, capable of growing in citrate medium or KCN broth and producing acid in inositol, salicin, or adonitol, does NOT belong to the Shigella group.

Any suspicious organism which conforms to the physiological and biochemical criteria listed above may be presumptively considered a member of the Shigella group. It is extremely hazardous to venture a decision based only on this limited information. Proceed to 11.14.

11.14 Serologic identification of presumptive Shigella - slide agglutination test

Test a 24 hour growth of the suspected Shigella organism from nutrient agar with Shigella somatic antisera poly groups A, B, C, D and Alkalescens-Dispar (A-D). Emulsify the

growth in 2 ml of 0.85% saline to make a heavy suspension in a serological test tube. Mark off six 1/2 inch squares with a wax pencil in a petri plate. In the upper left hand corner of each square, place a drop of the cell suspension. In the lower right hand corner place 0.05 ml of the antisera group A in the first box, group B in the second box, etc. In the sixth box place a drop of 0.85% NaCl. This is the autoagglutination control. Mix all cell suspensions with the respective antisera by means of a needle. Now rock the petri plate to enhance agglutination. Agglutination is usually not rapid and may take as long as 3-4 minutes to occur. A 4+ reaction will show a visible floc with the suspending fluid totally clarified.

A positive agglutination with group A, B, C, or D is presumptive evidence of the presence of Shigella. It is not a positive test for Shigella, nor should any final interpretive decision be made on the basis of this reaction. In the event of a negative agglutination reaction, it is advisable to boil the cell suspension for 30 minutes to destroy any interfering K antigen, which may be responsible and repeat the agglutination test once again with the boiled antigen.

11.15 Generic confirmation, and specific and serotypic identification

If the organism conforms to the biochemical criteria listed under 11.13 and agglutinates under the conditions of 11.14, or if it conforms to the biochemical conditions 11.13 but does not agglutinate 11.14, the organism should be further tested as to genus, species, and serotype.

12. DIRECT PLATING METHOD FOR THE DETECTION AND ENUMERATION OF STAPHYLOCOCCUS AUREUS OCCURRING IN FOOD

12.01 Equipment and materials

(a) sterile 100 x 15 mm petri dishes

(b) sterile, bent-glass streaking rods

(c) balance

(d) blender jars

(e) water bath, 37°C

(f) incubator, 35-37°C

12.02 Media and reagents

Trypticase or tryptic soy agar (40.35); Vogel-Johnson agar (40.38); brain heart infusion broth (BHI) (40.03); Butterfield's buffered phosphate diluent (41.03); desiccated coagulase plasma (rabbit) with EDTA (41.05).

12.03 Preparation of sample

Use balance with capacity of at least 2 kg and sensitivity of 0.1 g to aseptically weigh 50 g of unthawed (if frozen) sample into sterile high-speed blender jar. Add 450 ml of diluent, and blend 2 minutes. (If necessary to temper frozen sample to remove 50 g portion, hold not more than 18 hours at 2-5°C.)

Not more than 15 minutes should elapse from time sample is blended until all dilutions are in appropriate media.

If entire sample consists of less than 50 g, weigh portion equivalent to 1/2 sample and add amount of sterile diluent required to make 1:10 dilution. Total volume in blender jar must completely cover blades.

Prepare all decimal dilutions with 90 ml of sterile diluent plus 10 ml of previous dilution. Shake all dilutions 25 times in 1 footarc.

Pipets must deliver accurately the required volume. Do not use to deliver less than 10% of their total volume. For example, to deliver 1 ml, do not use pipet larger than 10 ml; to deliver 0.1 ml, do not use pipet larger than 1 ml.

12.04 Isolation and enumeration

Deliver 0.5 ml aliquots of decimal dilutions to duplicate plates of Vogel-Johnson agar. Distribute inoculum over surface of plates with sterile, bent-glass, streaking rods. When inoculum is completely absorbed by medium, invert plates and incubate 40-48 hours at 35-37°C. Select plates at the dilution having 20-200 well-distributed colonies. Plates from minimal dilution having less than 20 colonies may be

utilized. Count colonies (by groups of colonies having the same colonial appearance) not less than 1.0 mm in diameter which have reduced K_2TeO_3. Select at least one colony from each group and inoculate into 13 x 100 mm tube containing 0.2-0.3 ml of sterile brain heart infusion broth. Incubate at least 18 hours at 35-37°C. If a stock culture is desired, inoculate slant of trypticase soy agar and incubate 18-24 hours at 35-37°C. Store stock slants at 4°C.

To remaining brain heart infusion culture add 0.5 ml of reconstituted coagulase plasma containing EDTA and mix thoroughly. Incubate at 37°C and examine for clot formation at hourly intervals from 1 through 6 hours. Water bath incubation is preferable, but if adequate water bath space is not available, air incubation at 37°C is satisfactory. All cultures giving a positive coagulase reaction in 6 hours may be considered as Staph. aureus. Calculate number of Staph. aureus per gram of sample as follows:

> Add number of colonies represented by coagulase-positive cultures on duplicate plates of the test dilution; then multiply total number of Staph. aureus colonies by dilution factor to get number of Staph. aureus per gram of sample.

13. RAPID METHOD FOR THE RECOVERY OF ESCHERICHIA COLI[1/]

13.01 Introduction

This method should be used only on products where the presence of coliforms is not critical for the evaluation of the microbial flora, but it should not be used on those products for which the AOAC method is applicable.

13.02 Equipment and materials

(a) water bath, covered; 44.0°C ± 0.05°C

(b) blender

(c) balance

(d) pipets

(e) test tubes

13.03 Media and reagents

Lauryl sulfate tryptose broth (LST) (40.40); Levine's eosine methylene blue agar (EMB) (40.21); plate count agar (40.41); tryptone broth (40.17); MR-VP medium (40.16); Koser's citrate medium (40.15); Butterfield's buffered phosphate diluent (41.03); Kovac's reagent (41.07); alpha-naphthol solution (41.01); Voges-Proskauer reagent (41.15); methyl red solution (41.09).

[1/] Fishbein, et al. (11).

13.04 Preparation of sample

Prepare sample as directed in the method for the examination of frozen and/or prepared foods for the presence of coliforms, E. coli, aerobic plate count and coagulase-positive staphylococci. JAOAC 49 (1), 246-250 (1966).

13.05 Inoculation and incubation

Inoculate 3-tube most probable number (MPN) series into lauryl sulfate tryptose broth with 1 ml of inocula of 1:10, 1:100, 1:1000 dilutions, using triplicate tubes at each dilution. Incubate tubes in a covered water bath maintained at 44.0°C ± 0.05°C for 24 hours only.

13.06 Isolation

Streak all positive gassing tubes onto Levine's eosine methylene blue agar (EMB) and proceed as directed in the above referenced method (13.04).

Report results as E. coli MPN/g, RM (Rapid Method).

14. ISOLATION AND IDENTIFICATION OF VIBRIO PARAHAEMOLYTICUS

14.01 Introduction

Since 1958, Vibrio parahaemolyticus, a food-borne pathogen, has appeared to be a purely Japanese phenomenon. It caused numerous food-borne epidemics in Japan, accounting for well over 50% of all food poisonings or infections. The organism is a strict halophilic, marine vibrio, growing only in certain inland water areas where it successfully implants on marine forms such as fish and shellfish. When these are harvested and prepared for food, by mincing and serving in the raw state (Japanese style), the organism multiplies in great numbers and is the cause of food-borne epidemics. These epidemics occur in the summer season, and in the same prefectures near the Tokyo area, year after year.

In America, V. parahaemolyticus has already been isolated from Puget Sound waters as well as from marine products in the same area. The work has been accomplished in the laboratory of Dr. John Liston in the College of Fisheries at the University of Washington in the last two years. Much of this work has been carried out by Mr. John Baross in the

form of a graduate study. It is owing to the generous cooperation of Dr. Liston and Mr. Baross that we are able to use their salt water starch agar medium and other formulations, as well as some of their methodology in the following analytical procedure.

14.02 Suspect foods

Mr. Baross has recovered V. parahaemolyticus from oysters, Dungeness crabs, clams, and fish viscera (bottom feeders such as hake and English sole). The organism has also been recovered from marine and estuarine sediments as well as overlying waters. The Pacific oyster, Crassostrea gigas, and the Pacific butter clam may have counts as high as 10,000 vibrios per gram during the summer months. These counts will diminish to less than 10 per gram when the temperature drops to 8°C. This points to the fact that shellfish could well serve as a possible cause of gastroenteritis, particularly when consumed in the raw state. It is well to note that V. parahaemolyticus is not supposed to survive the frozen state, nor is it recoverable from marine environments when temperatures are much above 22°C.

14.03 Preview of the isolation and identification of Vibrio parahaemolyticus

The analytical approach is a parallel scheme employing the

Japanese methods, as exemplified in some degree by Sakazaki, and an American procedure utilizing the methods of Liston-Baross. The Japanese scheme, because it employs liquid enrichment media, answers the question, "Are any V. parahaemolyticus organisms present in the sample?" It is a qualitative approach. The Liston-Baross method answers the question, "How many V. parahaemolyticus organisms are present in the sample?" It is a quantitative approach. In the broadest sense, each method is both qualitative and quantitative at the same time. In the Japanese method, however, the ratio of the vibrios and competing flora will be altered permanently; in the Liston-Baross method, this ratio of natural types will more likely be fixed by the salt water starch agar plate method.

The Japanese isolation procedure employs a liquid enrichment phase consisting of two liquid broths, glucose-salt-Teepol broth and salt colistin broth. Both broths are inoculated in parallel with the sample. This is followed by streaking onto an isolation agar, thiosulfate-citrate-bile salts-sucrose agar. The isolated colonies from the agar are thereafter characterized biochemically in accordance with the standard classical Japanese data.

The Liston-Baross method employs no liquid enrichment phase. Instead, the sample is plated directly, by the spread plate technic, onto an isolation agar, salt water starch agar. This medium is cultured anaerobically for 2-3 days. The isolated colonies from this agar are thereafter characterized biochemically in accordance with the standard classical Japanese data.

Because there is no certain serological support for the V. parahaemolyticus isolates at the present time, it is safest to complete the rather lengthy biochemical identification scheme, to assure the correct identification of genus and species. There is no complete agreement among authors as to what constitutes the primary criteria for identification of V. parahaemolyticus. Accordingly, we have listed the biochemical characterization (standard classical Japanese data) under Differential Tests I, II, and III. Differential Test I is a resume of the Liston-Baross approach wherein emphasis is placed on pleomorphism and hemolysis. These are criteria which are not stressed by the Japanese, who tend to rely on tests such as TSI, V-P, halophilism, nitrate reduction, etc. A table is included

to separate two possible interfering types of nonenteropathogenic vibrios, V. alginolyticus and V. anguillarum, from the enteropathogen, V. parahaemolyticus.

14.04 Equipment and materials

(a) 35°C incubator

(b) sterile glass rods

(c) blender and sterile jars

(d) Case laboratories anaerobic apparatus and nitrogen gas cylinder or any equivalent anaerobic device

(e) pipets

(f) dilution blanks

(g) sterile wide-mouth screwcap jars, pint size

(h) phase microscope

(i) Millipore filter apparatus

14.05 Media and reagents

Artificial sea water (14.20.01); B-sea water broth (BSWB) (14.20.02); blood agar (14.20.03); carbohydrate media (14.20.04); cholera red broth (14.20.05); glucose salt Teepol broth (GSTB) (14.20.06); Hugh-Liefson glucose broth (HLGB) (14.20.07); Koser's citrate medium (14.20.08); malonate broth (14.20.09); motility agar (14.20.10); MR-VP

medium (14.20.11); nitrate broth (14.20.12); nutrient gelatin (14.20.13); phenylalanine agar (14.20.14); salt colistin broth (SCB) (14.20.15); salt trypticase broth (STB) (14.20.16); salt water starch agar (SWSA) (14.20.17); thiosulfate-citrate-bile salts-sucrose agar (TCBS) (14.20.18); triple sugar iron agar (TSI) (14.20.19); trypticase or tryptic soy agar, 3% NaCl (STSI) (14.20.20); tryptone broth (14.20.21); urea broth (14.20.22); alpha-naphthol solution (14.21.01); phenylenediamine solution (14.21.02); dilution water, 3% NaCl (14.21.03); ferric chloride solution (14.21.04); hydrogen peroxide solution (14.21.05); sulfanilic acid solution (14.21.06); alpha-naphthylamine solution (14.21.07); creatine, crystalline; petrolatum, sterile; sulfuric acid, concentrated; zinc powder.

14.06 Enrichment in liquid broth (parallel broth culture) (Japanese method)

Weigh out 50 grams of the shellfish sample into 450 ml of 3% NaCl dilution water in a Waring Blendor jar, and blend for 3 minutes. This represents a 1:10 dilution. From this blended sample,[1/] make 1:100, 1:1000, 1:10,000 dilutions,

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[1/] In the examination of marine waters the range 1:10-1:10,000 dilution is tested, and the 1:10 concentration is cultured directly into 2x strength broths.

using appropriate dilution blanks. Inoculate 10 ml of the 1:10 dilution into GSTB-2x concentration and into SCB-2x concentration. This constitutes the 1:1 dilution. Inoculate the remaining dilutions, 1:10, 1:100, 1:1000, and 1:10,000, into single strength GSTB and into single strength SCB. Incubate the broth tubes overnight (18 hours or less) at 35°C, and thereafter streak a loopful of the culture onto the selective agars. All the prepared dilutions are saved for examination by the Liston-Baross isolation method [14.07 (a)].

14.07 Isolation phase (parallel Liston-Baross and Japanese methods)

(a) Liston-Baross method. From each of the prepared dilutions described in 14.06, pipet a 0.1 ml portion onto SWSA plates and streak the material, using the spread plate technic. Incubate the plates anaerobically for 48-72 hours at 35°C. Anaerobiosis may be obtained by using the Case anaerobic apparatus, evacuating to negative 20 lb, and flushing with nitrogen gas for a total of four times. Other equivalent technics may be employed to establish anaerobic conditions. On this medium Vibrio parahaemolyticus will appear as white, non-spreading colonies, which will be amylase positive (indicated by the presence of a halo around the colony).

Subculture such colonies onto the following media: TSI, B-sea water broth (BSWB), trypticase or tryptic soy agar, blood agar, and motility agar (14.09).

Second Day

(b) Japanese method. Streak a 3 mm loopful of the 18 hour culture from the GSTB and the SCB broths in Section 14.06 onto thiosulfate-citrate-bile salts-sucrose agar (TCBS). Incubate the TCBS agar at 35°C for 18 hours. On this medium, V. parahaemolyticus colonies are round, 2-3 mm in diameter, with a green or blue center. V. alginolyticus, a non-desirable type, will appear larger and yellow. Coliforms, Proteus, and enterococci may also grow on the medium but these will be very small and translucent colonies, easily distinguished from V. parahaemolyticus. Subculture suspicious colonies from TCBS agar onto the following media: TSI, B-sea water broth (BSWB), trypticase soy agar, blood agar, and motility agar (14.08).

Third Day

14.08 Differential Test I

In the isolation test (14.06), the suspicious colonies on SWSA will have had 2 days' incubation. In 14.07 (b), the suspicious colonies on TCBS agar will have had 18 hours of incubation. Subculture the suspicious colonies from both

isolation agars as follows:

(a) TSI. Streak the slant and stab the butt. Incubate the medium overnight at 35°C. V. parahaemolyticus produces an acid butt and an alkaline slant. It produces no gas and is a negative H_2S producer.

(b) BSWB. Inoculate and incubate this broth at 35°C overnight. This culture serves as inoculum for other tests, as well as for motility studies, the Gram stain, and pleomorphism observation under the phase microscope. V. parahaemolyticus is a motile, Gram-negative, pleomorphic organism exhibiting short and long rods, as well as spheres and spirals.

(c) Salt trypticase soy agar (STSA). Inoculate a slant of this medium and incubate at 35°C for 24 hours. This will serve as inoculum and holding medium.

(d) Blood agar. Streak a blood agar plate and incubate at 35°C for 24 hours. V. parahaemolyticus produces a positive beta hemolysis reaction (clear transparent zone). This test should be performed on freshly isolated organisms, as this property is lost with repeated subculture of the organism.

(e) Motility agar. Inoculate a tube of motility agar by stabbing the column of the medium to a depth of approximately 5 mm. Incubate for 24 hours at 35°C. A circular growth from the line of stab constitutes a positive test. This test for motility may be used in lieu of a motility test (hanging drop) from BSWB.

Fourth Day

14.09 Differential Test II

Organisms which are found to be motile, hemolytic, pleomorphic, Gram-negative bacteria, which produce an acid butt and alkaline slant on TSI and are negative for H_2S production, are tested further as follows:

(a) Salt trypticase broth (STB), halophilism. Inoculate four tubes of STB base containing 0, 3%, 7%, and 10% NaCl concentrations from STSA slants and incubate at 35°C for 24 hours. V. parahaemolyticus will grow well in 3% and 7% NaCl concentrations in this medium but will not grow in 0 and 10% NaCl concentrations.

(b) Nitrate broth. Inoculate a tube of nitrate broth from STSA slants. Incubate for 24 hours at 35°C. Prior to testing for reduction of nitrate to nitrite, mix equal amounts of sulfanilic acid solution and alpha-naphthylamine

solutions, and add 0.1 ml of this mixture to the culture tube. If nitrate has been reduced to nitrite, a red color will develop. V. parahaemolyticus reduces nitrate to nitrite.

It is wise to confirm negative tests by the addition of a pinch of zinc dust. The production of a red color indicates the presence of unreduced nitrate. In the case of equivocal results, cultures may be incubated up to 4 days.

(c) Nutrient gelatin. Inoculate a tube of nutrient gelatin from an STSA slant by means of a needle and incubate at 35°C from 1-7 days. To test for proteolysis, cool the medium below 20°C; if the medium does not resolidify, the gelatin has liquefied. V. parahaemolyticus is a rapid gelatin liquefier.

(d) Hugh-Liefson glucose broth (HLGB). Using an STSA inoculum, stab 2 tubes of Hugh-Liefson glucose broth with a needle. Overlay one tube with melted sterile petrolatum to a depth of about 1 inch. Incubate at 35°C for 2 days, or longer if necessary. If the organism changes the color in both tubes from purple to yellow, it is a carbohydrate fermenter. But if the yellow color change occurs only in the open tube, it is a carbohydrate oxidizer. V. parahaemolyticus is a positive glucose fermenter, producing no gas.

(e) Cytochrome oxidase test. This test may be performed on any discard plate or slant with growth on it such as blood agar or STSA slant. Two or three drops of the alpha-naphthol solution are allowed to run down the slant or over the colony on a plate. This is followed by an equal amount of the "phenylenediamine" solution. A positive reaction is shown by the rapid development of a dark blue color within 2 minutes. V. parahaemolyticus is positive by this test.

(f) Indole test (tryptone broth). To perform the indole test, inoculate tryptone broth from an STSA slant. Incubate the culture at 35°C for 24 hours. Test for indole production by adding 0.5 ml of Kovac's reagent to the tube and shaking gently. A positive test is indicated by the formation of a deep red color. V. parahaemolyticus is indole positive.

(g) Methyl red test (MR-VP medium). To perform the methyl red test, inoculate MR-VP medium from an STSA slant. Incubate at 35°C for 4 days. Add a few drops of methyl red solution to 5 ml of the culture. A distinct red color (acid reaction) constitutes a positive reaction; a yellow color is negative. V. parahaemolyticus is methyl red-positive.

(h) Voges-Proskauer test (MR-VP medium). Inoculate a tube of MR-VP medium from an STSA slant and incubate at 35°C for 48 hours. To 1 ml of this culture add 0.2 ml of 40% KOH solution, shake well, and add 0.6 ml of 5% alpha-naphthol solution. Add a few crystals of creatine and shake well. A red color forming within 2-4 hours constitutes a positive V-P test. V. parahaemolyticus is V-P negative.

(i) Carbohydrate fermentation-cellibiose and sucrose broths. Inoculate a tube of cellibiose and sucrose broths from an STSA slant. Incubate at 35°C for 4 or 5 days. A negative reaction will leave the culture tube with its original purple color unchanged. Acid production will cause a change in color from purple to yellow. V. parahaemolyticus will not produce an acid reaction in cellibiose within the first 24 hours. Sakazaki states that it is likewise negative in sucrose. However, Liston and Baross report that there may be an element of uncertainty with regard to the sucrose reaction; it may be negative or may produce acid slowly.

14.10 Differential Test III

(a) Citrate utilization. Inoculate a tube of Koser's citrate medium from an STSA slant culture, effecting the

barest transfer of cells to the citrate medium by inoculating the side of the tube just below the surface of the medium. Incubate at 35°C for 4 or 5 days. A positive test consists of visible, turbid growth in the medium. V. parahaemolyticus is positive.

(b) Phenylalanine deaminase. Inoculate a tube of phenylalanine agar with a fairly heavy inoculum from an STSA slant culture. Incubate at 35°C for 24 hours. Following incubation, allow 4 or 5 drops of ferric chloride reagent to run down the growth on the slant. A positive reaction is denoted by the development of a green color in the syneresis fluid and in the agar slant, indicating that phenylalanine has been deaminated to phenylpyruvic acid. V. parahaemolyticus gives a negative reaction in this test.

(c) Urease test. Inoculate a tube of urea broth from an STSA slant. Incubate at 35°C for 24 hours. Cultures producing a red color (alkaline reaction) are positive for urease production. V. parahaemolyticus is urease-negative.

(d) Malonate utilization. Inoculate a tube of malonate broth by means of a 3 mm loopful from a 24-hour culture of BSWB. Incubate at 35°C for 48 hours. A positive reaction is indicated by a change in the color of the indicator from

green to dark blue. V. parahaemolyticus gives a negative reaction in this test.

(e) Catalase test. Pour a few drops of hydrogen peroxide solution on the agar slope surface of a 24-hour culture of a tube of STSA. If catalase is present, bubbles of oxygen will be released from the surface of the growth. V. parahaemolyticus is catalase-positive.

(f) Cholera-red reaction. Inoculate a tube of cholera-red medium from an STSA slant. Incubate at 35°C for 24 hours. The development of a pink to violet color upon the addition of 2-3 drops of concentrated sulfuric acid is a positive test. (This test is based on the simultaneous production of indole from tryptophane and nitrite from nitrate by the test organism. The addition of sulfuric acid to the culture results in the production of a nitroso-indole, which yields a pink to violet color.) V. parahaemolyticus gives a variable response with this test.

(g) Additional carbohydrate fermentations. Media inoculations and incubation are as previously listed under 14.09 - Differential Test II - (i). The following sugar broths yield a positive fermentation reaction with V. parahaemolyticus: maltose, mannitol, and trehalose. The following sugar broths yield a negative fermentation reaction with V. parahaemolyticus: lactose, xylose, adonitol, dulcitol, inositol, sorbitol, and salicin.

V. parahaemolyticus yields a variable fermentation response in arabinose broth.

14.11 Recapitulation of the identifying characters of V. parahaemolyticus.

Gram stain	Gram-negative
pleomorphism	positive
motility	motile
blood agar	B-hemolysis
TSI	acid butt, alkaline slant
salt trypticase broth--3%, 7%	positive
salt trypticase broth--0, 10%	negative
indole	positive
methyl red	positive
V-P	negative
citrate utilization	positive
H_2S production	negative
phenylalanine deaminase	negative
nitrate	reduced
urease	negative
gelatin	liquefied
malonate	negative
catalase	positive
cytochrome oxidase	positive
cholera-red	variable

Hugh-Liefson glucose	positive fermentation without gas
cellibiose	negative fermentation within 24 hours
sucrose	not fermented or very slowly
arabinose	variable
maltose	fermented
mannitol	fermented
trehalose	fermented
lactose	not fermented
rhamnose	not fermented
xylose	not fermented
adonitol	not fermented
inositol	not fermented
sorbitol	not fermented
salicin	not fermented

14.12 At times it may be necessary to distinguish the two non-enteropathogenic types, V. alginolyticus and V. anguillarum, from the enteropathogenic V. parahaemolyticus. The following table indicates how the resolution of the 3 vibrios may be effected.

Characteristic	V. parahaemolyticus	V. alginolyticus	V. anguillarum
7% NaCl trypticase broth	+	+	-
10% NaCl trypticase broth	-	+	-
Voges-Proskauer reaction	-	+	-
Sucrose fermentation	-	+	±
Cellibiose fermentation (24 hours)	-	-	+

14.13 Serology

Serological identification of the various serotypes of V. parahaemolyticus is in such a state of uncertainty as to preclude its role as a diagnostic tool at the present time.

14.14 Interpretation of positive findings of V. parahaemolyticus

V. parahaemolyticus is not an infective agent for man. A person ill with this organism will not infect a healthy individual. Furthermore, the Japanese have found that large numbers of the organism must be present before the Shigella-like syndromes occur after ingestion of the food. Thus, the kind of food,

the numbers of V. parahaemolyticus present, the type of market and consumer handling, the epidemiological evidence, and the ecological data must all be considered when interpreting the health hazard potential of a food sample.

14.20 MEDIA

Artificial sea water.....14.20.01
B-sea water broth (BSWB).....14.20.02
Blood agar.....14.20.03
Carbohydrate media.....14.20.04
Cholera red broth.....14.20.05
Glucose salt Teepol broth (GSTB).....14.20.06
Hugh-Liefson glucose broth (HLGB).....14.20.07
Koser's citrate medium.....14.20.08
Malonate broth.....14.20.09
Motility agar.....14.20.10
MR-VP medium.....14.20.11
Nitrate broth.....14.20.12
Nutrient gelatin.....14.20.13
Phenylalanine agar.....14.20.14
Salt colistin broth (SCB).....14.20.15
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14.20.01 Artificial Sea Water (Sakazaki)

NaCl	23.4 g
KCl	66.4 g
Na_2SO_4	3.9 g
$NaHCO_3$	19.3 g
$MgCl_2$	4.9 g
Distilled H_2O	500 ml

Sterilize medium separately at 121°C for 15 minutes and adjust pH to 7.0. Most of the media described in this section are prepared in a 4% NaCl solution, which is a substitute for natural aged and filtered sea water. To use the artificial sea water formulation in a particular medium, it is only necessary to compound the particular medium in 500 ml of distilled water, exclude the 4% NaCl, and add 500 ml of the artificial sea water.

14.20.02 B-Sea Water Broth (Liston-Baross) (BSWB)

Peptone (Difco)	4 g
Phytone (BBL)	4 g
Yeast extract (Difco)	1 g
NaCl	40 g
Distilled H_2O	1 L

Aged, filtered sea water, if available, is preferable to the 4% NaCl solution which has been substituted for it. Final pH 7.5. Sterilize at 121°C for 15 minutes.

14.20.03 Blood Agar

Bacto blood agar base	40 g
NaCl	20 g
Distilled H_2O	1 L

Dispense the salt blood agar base in flasks of known volume and autoclave at 15 lbs. pressure (121°C) for 15 minutes. Adjust the pH to 7.0. Cool to 45-50°C and, while it is in the liquid state, add 5% sterile defibrinated blood aseptically, thoroughly mix, and pour into the petri plates.

14.20.04 Carbohydrate Media

BAM formula (40.05) is changed to include 30 g of NaCl per liter of the medium. Sterilize at 121°C for 10 minutes. Final pH 7.0.

All other indications and contraindications of (40.05) should be observed. If there is any doubt about the stability of the carbohydrate to the sterilization process, it is best to Millipore-filter sterilize a concentrated solution of it and add appropriate amounts to the previously sterilized basal medium.

14.20.05 Cholera Red Broth

Peptone	10 g
NaCl	25 g
$NaNO_3$	0.01 g
Distilled H_2O	1 L

Adjust to pH 7.2, dispense in 5 ml portions, and sterilize at 121°C for 15 minutes.

14.20.06 Glucose Salt Teepol Broth (GSTB)

	Single Strength	Double Strength
Beef extract	3 g	6 g
Peptone	10 g	20 g
NaCl	30 g	60 g
Glucose	5 g	10 g
Methyl violet	0.002 g	0.004 g
Teepol[1/]	4 ml	8 ml
Distilled H_2O	1 L	1 L

Dispense the single strength broth in 10 ml portions. Dispense the double strength broth in 20 ml portions in tubes large enough to accommodate a 10 ml charge of sample. If larger quantities of sample are to be examined (25 g), use screwcap jars containing 225 ml of single strength broth. Sterilize at 121°C for 15 minutes. Final pH 9.4.

[1/] Teepol is available through Mr. George Moore, Shell Chemical Company, 110 West 51st Street, New York, N. Y. 10020. One gallon containing 9 pounds costs $4.65.

14.20.07 Hugh-Liefson Glucose Broth (Liston-Baross) (HLGB)

Peptone	2 g
Yeast extract (Difco)	0.5 g
NaCl	38.0 g
Glucose	10 g
Bromcresol purple	0.015 g
Agar	3 g
Distilled H_2O	1 L

Adjust the pH to 7.4 before autoclaving at 121°C for 15 minutes. By excluding glucose and adding 10 ml of a 10% solution of sterile sucrose, cellibiose, or lactose to the above basal medium, these carbohydrates may be tested by the Hugh-Liefson procedure, in consonance with the data of some Japanese workers.

14.20.08 Koser's Citrate Medium

To BAM formula (40.15) add 30 g of NaCl per liter of the medium. Sterilize at 121°C for 15 minutes. Final pH 6.7.

14.20.09 Malonate Broth

To BAM formula (40.23) add 30 g of NaCl per liter of the medium. Sterilize at 121°C for 15 minutes. Final pH 6.7.

14.20.10 Motility Agar

To BAM formula (40.26) add an additional 15 g of NaCl per liter of medium. Sterilize at 121°C for 15 minutes. Final pH 7.2.

14.20.11 MR-VP Medium

To BAM formula (40.16) add 30 g of NaCl per liter of the medium. Sterilize at 121°C for 15 minutes. If the commercial dehydrated medium is employed, do not adjust pH.

14.20.12 **Nitrate Broth**

Meat extract	3 g
Peptone	5 g
KNO_3 (nitrite free)	1 g
NaCl	30 g
Distilled H_2O	1 L

Sterilize at 121°C for 15 minutes. Final pH 7.0.

14.20.13 **Nutrient Gelatin**

Beef extract	3 g
Peptone	5 g
Gelatin	120 g
NaCl	30 g
Distilled H_2O	1 L

Sterilize at 121°C for 12 minutes. Final pH 7.0.

14.20.14 Phenylalanine Agar

Yeast extract	2 g
DL-phenylalanine	
or	
L-phenylalanine	1 g
Na_2HPO_4	1 g
NaCl	25 g
Agar	12 g
Distilled H_2O	1 L

Tube and sterilize at 121°C for 10 minutes and allow to solidify in a long slanted position.

Final pH 7.3.

14.20.15 Salt Colistin Broth[2/] (SCB)

	Single Strength	Double Strength
Yeast extract	3 g	6 g
Peptone	10 g	20 g
NaCl	20 g	40 g
Colistin methansulfonate	500 µg/ml	1000 µg/ml
Distilled H_2O	1 L	1 L

Final pH 7.4. This medium should not be heated.

The volume relationships are identical with those listed under 14.20.06 .

[2/] SCB is available in dehydrated form from Eiken (Nihon Eiyo Kagaku) Co. Ltd., 8-33, 1 Hongo, Bunkyo-Ku, Tokyo, Japan.

14.20.16 Salt Trypticase Broth (Liston-Baross) (STB)

Trypticase or tryptone	10 g
Yeast extract (Difco)	2 g
Distilled H_2O	1 L

Add 0, 30, 70, 100 g of NaCl per liter to the above medium to make, respectively, 0, 3, 7, and 10% NaCl trypticase broth for salt tolerance tests (halophilism).

Sterilize at 121°C for 15 minutes. Final pH 7.5.

14.20.17 Salt Water Starch Agar (Liston-Baross) (SWSA)

Peptone	3 g
Yeast extract	1 g
Soluble potato starch	5 g
Agar	15 g
NaCl	40 g
Distilled H_2O	1 L

Final pH 7.5-7.8. Sterilize at 121°C for 15 minutes. Where an incidence of Bacilli is anticipated, the addition of 2 units of Penicillin G per ml of medium is recommended.

14.20.18 Thiosulfate-Citrate-Bile Salts-Sucrose Agar (TCBS)

Yeast extract	5 g
Peptone	10 g
Sucrose	20 g
Sodium thiosulfate (5 H_2O)	10 g
Sodium citrate (2 H_2O)	10 g
Sodium cholate	3 g
Ox gall	5 g
Sodium chloride	10 g
Ferric citrate	1 g
Bromthymol blue	0.04 g
Thymol blue	0.04 g
Agar	15 g
Distilled H_2O	1 L

Final pH 8.6. This medium should <u>not</u> be autoclaved. It is available commercially in dehydrated form from BBL , Difco, and Eiken.

14.20.19 Triple Sugar Iron Agar (TSI)

To BAM formula (40.33) add 25 g NaCl per liter of medium. Sterilize at 121°C for 15 minutes. Final pH 7.4.

14.20.20 Trypticase or Tryptic Soy Agar (TSA) 3% Salt

Trypticase or tryptone	15 g
Phytone or soytone	5 g
NaCl	30 g
Agar	15 g
Distilled H_2O	1 L

Sterilize at 121°C for 15 minutes. Final pH 7.3.

14.20.21 Tryptone Broth

To BAM formula (40.17) add 30 g of NaCl per liter of the medium. Sterilize at 121°C for 15 minutes. Final pH 7.0.

14.20.22 Urea Broth

To BAM formula (40.37) add 30 g of NaCl per liter of the medium. Filter-sterilize and aseptically distribute the medium into tubes. Final pH 6.8.

Cytochrome Oxidase Reagents

14.21.01 alpha-Naphthol Solution

alpha-Naphthol	1 g
Ethyl alcohol	100 ml

14.21.02 "Phenylenediamine" Solution

N,N-dimethyl-p-phenylene-diamine dihydrochloride	1 g
Distilled H_2O	100 ml

14.21.03 3% NaCl Dilution Water

NaCl	30 g
Distilled H_2O	1 L

This formula is employed for both sample dilution and blanks. Sterilize at 121°C for 15 minutes. Final pH 7.0.

Nitrite Test Reagents.

14.21.04 Sulfanilic Acid Solution

Sulfanilic acid	0.8 g
5 N acetic acid	100 ml

14.21.05 alpha-Naphthylamine Solution

alpha-Naphthylamine	0.5 g
5 N acetic acid	100 ml

To make 5 N acetic acid, add 28.75 ml of glacial acetic acid to 71.25 ml of distilled water.

14.21.06 Hydrogen Peroxide Solution

30% H_2O_2	33 ml
Distilled H_2O	67 ml

14.21.07 Ferric Chloride Solution

$FeCl_3$	10 g
Distilled H_2O	100 ml

40. MEDIA

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General Instructions

Ingredients and reagents used to prepare the following media may be the product of any manufacturer if comparative tests have shown that satisfactory results are obtained. Carbohydrates must be chemically pure and suitable for biological use; inorganic chemicals must be ACS reagent grade; and dyes must be certified by "Biological Stain Commission" for use in media.

For convenience, dehydrated media of any brand equivalent to the formulation may be used unless instructions indicate otherwise. Test each lot of prepared medium for sterility and growth-promoting qualities of suitable organisms (e.g., inoculate media containing lactose with coliforms; Staphylococcus media with Staphylococcus, etc.).

Determine hydrogen ion concentrations (pH), using an electronic pH meter which is standardized against known buffers, prepared according to AOAC (latest edition). Adjust pH when necessary by adding sufficient 1 N sodium hydroxide or 1 N hydrochloric acid.

Unless otherwise indicated, sterilize media by steam under pressure at 121°C (15 lbs.) for 15 minutes.

An asterisk (*) beside the medium name means that the dehydrated prepared medium is not available commercially.

40.01 **Acid Broth***

Proteose peptone	5 g
Yeast extract	5 g
Dextrose	5 g
K_2HPO_4	4 g
Distilled H_2O	1 L

Adjust to pH 5.0. Dispense into tubes.

40.02 Anaerobic egg agar* .

3 fresh eggs	
Yeast extract	5 g
Tryptone	5 g
Proteose peptone	20 g
NaCl	5 g
Na thioglycollate	1 g
Agar	20 g
Distilled H_2O	1 L

Wash eggs with a stiff brush, and drain. Soak them in 70% ethyl alcohol 10-15 minutes, remove them and ignite the adhering alcohol. Crack them aseptically. Separate and discard the whites. Drain the contents of the yolk sacs into a sterile stoppered graduate, and discard the sacs. Add to the yolk material an equal volume of sterile saline. Invert the cylinder several times to mix. Combine the remainder of the ingredients in the water, dissolve, dispense, and sterilize. To 1 L of the melted agar at 45-50°C, add 80 ml of the yolk-saline mixture. Mix and pour plates immediately. Dry plates at room temperature 2-3 days, or at 35°C for 24 hours. Discard contaminated plates. Store sterile plates in the refrigerator.

40.03 **Brain heart infusion broth (BHI)**

Calf brain, infusion from	200.0 g
Beef heart, infusion from	250.0 g
Proteose peptone or Gelysate	10.0 g
NaCl	5.0 g
$Na_2HPO_4.12H_2O$	2.5 g
Dextrose	2.0 g

Dissolve ingredients in 1 L H_2O, heating gently if necessary. Dispense in bottles or tubes for storage, and autoclave 15 minutes at 121°C. Final pH 7.4 ± 0.1.

40.04 Bromcresol Purple Dextrose Broth (BCP)*

Dextrose	10 g
Beef extract	3 g
Peptone	5 g
Bromcresol purple, 1.6% in alcohol	2 ml
Distilled H_2O	1 L

Adjust to pH 7.0. Dispense into tubes.

40.05 Carbohydrate Fermentation Broths

Peptone	10 g
Meat extract	3 g
Sodium chloride	5 g
Carbohydrate	see below
Bromcresol purple	0.04 g
Distilled H_2O	1 L

Carbohydrates - 10 g/L: glucose, lactose, sucrose, mannitol.
5 g/L: salicin, inositol, adonitol, maltose, arabinose, xylose.

The following sugars may be added to the media prior to sterilization: glucose, mannitol, salicin, adonitol, and inositol. The following sugars are prepared in 10% solutions in distilled water, sterilized by filtration, and appropriate amounts added to previously sterilized basal media: lactose, sucrose, maltose, arabinose and xylose.

Dispense all carbohydrates into tubes containing inverted fermentation tubes.

40.06 Chopped Liver Broth*

Fresh beef liver	500 g
Distilled H_2O	800 ml
Peptone	10 g
K_2HPO_4	1 g
Soluble starch	1 g

Grind the liver into the water. Heat it to the boiling point, and permit it to simmer 1 hour. Cool, adjust to pH 7, and boil 10 minutes. Filter through cheesecloth, pressing out the excess liquid. To the broth add peptone, K_2HPO_4 and soluble starch. Adjust to pH 7. Bring the volume of broth to 1 L with distilled water. Filter it through coarse filter paper. (At this point the broth and meat can be stored separately in the freezer for future use.) To 18 or 20 mm test tubes add 1/2 to 1 inch of the chopped liver, and 10 to 12 ml of broth. Autoclave for 20 minutes at 121°C.

40.07 Cooked Meat Medium

Beef heart	454 g
Proteose peptone	20 g
Dextrose	2 g
Sodium chloride	5 g

Suspend 12.5 g of commercial dehydrated cooked meat medium in 100 ml of cold distilled water. Mix thoroughly and allow to stand for 15 minutes until all particles are thoroughly wetted. Medium may also be prepared by distributing 1.25 g into test tubes, adding 10 ml of cold distilled water, and mixing thoroughly, letting it stand to insure thorough wetting of all particles.

Sterilize for 15 minutes at 121°C. Final pH 7.2.

40.08 Desoxycholate Citrate Agar (DC)

Meat infusion (pork, beef, or beef heart)	330 g
Peptone	10 g
Lactose	10 g
Sodium citrate	20 g
Sodium desoxycholate	5 g
Ferric ammonium citrate	2 g
Agar	20 g
Neutral red	0.02 g
Distilled H_2O	1 L

Heat to boiling to dissolve the medium completely. Do not sterilize in the autoclave. Pour plates and allow the surface to dry for 2 hours with covers partially removed, before inoculation. This is the Difco product. Final pH 7.2.

40.09 Distilled Water (sterile, 200 ml)

Dispense 200 ml portion of distilled water into 500 ml flask or screwcap pint jar; plug or cap loosely. Autoclave 15 minutes at 121°C. Cool before using.

40.10 Distilled Water (sterile, 1000 ml)

Dispense 1000 ml portion of distilled water into 2 L wide-mouth flask or wide-mouth jar; plug or cap loosely. Autoclave 20 minutes at 121°C. Cool before using.

40.11 Ellner's Sporulation Broth*

Polypeptone (BBL) - Pantone (Difco)	10 g
Yeast extract	3 g
Soluble starch	3 g
$MgSO_4$	0.1 g
KH_2PO_4	1.5 g
$Na_2HPO_4.7H_2O$	50 g
Distilled H_2O	1000 ml

Dissolve salts and other ingredients separately; then combine. Adjust to pH 7.2. Dispense 15 ml in 20 x 150 mm tubes and autoclave at 121°C for 15 minutes.

40.12 **Fluid Thioglycollate Medium**

L-Cystine	0.5 g
Agar	0.75 g
Sodium chloride	2.5 g
Dextrose	5.5 g
Yeast extract	5.0 g
Pancreatic digest of casein	15.0 g
Sodium thioglycollate	0.5 g
Resazurin	0.001 g
Distilled H_2O	1.0 L

Final pH 7.1 $\pm$ 0.1.

40.13 Fluid Thioglycollate (Azolectin-Tween Modification)*

Dehydrated prepared fluid thioglycollate medium (or ingredients as in USP)	29.5 g
Azolectin (available from Associated Concentrates Inc., 57-01 32nd Ave., Woodside, N. Y.)	5 g
Tween 20 (available from Atlas Powder Co., Wilmington, Del.)	40 ml
Distilled H_2O	1 L

Dissolve the azolectin in 340 ml of cold distilled water. Dissolve the Tween 20 in 360 ml of distilled water by holding at the boiling point for several minutes. Dissolve the dehydrated prepared fluid thioglycollate medium in 300 ml of distilled water. Combine the three solutions. Adjust to pH 7.7 before sterilization. Autoclave for 20 minutes at 121°C. Final pH 7.1 or 7.2. Dispense according to USP instructions.

40.14 Gram-Negative Broth (GN)

Polypeptone (BBL) - Pantone (DIFCO)	20 g
Dextrose	1 g
d-Mannitol	2 g
Sodium citrate	5 g
Sodium desoxycholate	0.5 g
Dipotassium phosphate	4 g
Monopotassium phosphate	1.5 g
Sodium chloride	5 g
Distilled H_2O	1 L

Dispense into tubes/screwcap jars and autoclave for 15 minutes at 10 lbs. pressure (116°C) or steam sterilize for 30 minutes. Avoid overheating.

Final pH 7.0 $\pm$ 0.1.

IMVC Media

40.15 Koser's Citrate Medium

Sodium ammonium phosphate	1.5 g
Monopotassium phosphate	1.0 g
Magnesium sulfate	0.2 g
Sodium citrate	3.0 g
Distilled H_2O	1.0 L

Dispense into tubes. Final pH 6.7 ± 0.1.

40.16 MR-VP Medium

Polypeptone (BBL) or buffered peptone (DIFCO)	7.0 g
Dextrose	5.0 g
K_2HPO_4	5.0 g
Distilled H_2O	1.0 L

Dispense into tubes. Final pH 6.9 ± 0.1.

40.17 Tryptone Broth

Tryptone (DIFCO) or trypticase (BBL)	10.0 g
Distilled H_2O	1.0 L

Dispense into tubes.

40.18 Indole Nitrite Medium

Trypticase	20 g
Na_2HPO_4	2 g
Dextrose	1 g
KNO_3	1 g
Agar	1 g
Distilled H_2O	1000 ml

(Available dehydrated from BBL)

Heat to dissolve agar, adjust to pH 7.2, mix thoroughly, and dispense 11 ml in 16 x 150 mm tubes. Autoclave at 121°C for 15 minutes. (Add 2 g of agar per liter if desired.)

40.19 Iron Milk Medium*

Fresh whole milk

Small nails or steel wool (not stainless)

Heat nails in a Bunsen flame to remove oil or coatings. Wash steel wool with detergent and rinse. Add pea-sized ball of steel wool or a nail to each 20 x 150 mm tube and fill with 15 ml of milk. Autoclave at 121°C for 5-6 minutes.

40.20 KCN Broth (Caution! KCN is a deadly poison.)

Basal broth (DIFCO):

Proteose peptone No. 3	3 g
Disodium phosphate	5.64 g
Monopotassium phosphate	0.225 g
Sodium chloride	5 g
Distilled H_2O	1 L

Sterilize in the autoclave in 100 ml portions. Cool and store at 5-8°C. Final pH 7.6 ± 0.1. Prepare 0.5% KCN with cold (5-8°C) sterile distilled water. Using a sterile syringe or bulb pipet (do not pipet by mouth), add 1.5 ml of cold KCN solution to each 100 ml of cold sterile basal broth. Distribute 1-1.5 ml to sterile 13 x 100 mm test tubes and stopper quickly with No. 2 corks impregnated with paraffin. Prepare corks by boiling in paraffin ca 5 minutes. Store the finished medium in the refrigerator not more than 2 weeks. Final pH 7.6.

40.21 Levine's Eosine Methylene Blue Agar (Levine's EMB)

Peptone	10 g
Lactose	10 g
K_2HPO_4	2 g
Agar	15 g
Eosin Y	0.4 g
Methylene blue	0.065 g
Distilled H_2O	1 L

Heat to boiling with frequent agitation until the ingredients dissolve. Dispense into flasks or bottles for storage. Autoclave for 15 minutes at 121°C. Final pH 7.1 ± 0.1. On remelting the agar, resuspend the precipitate by gentle agitation before pouring plates.

40.22 Liver Veal Egg Yolk Agar (LVEY)*

3 fresh eggs (antibiotic-free)

1 L melted sterile Difco liver veal agar

0.1% mercuric chloride solution

70% ethyl alcohol

Wash eggs with a stiff brush and drain. Soak eggs in 0.1% mercuric chloride solution for 1 hour. Pour off mercuric chloride solution and replace with 70% ethyl alcohol. Soak in 70% ethyl alcohol for 30 minutes. Crack the eggs aseptically and discard the whites. Remove the yolk with a 50 ml Luer-lok syringe. Place in a sterile container and add an equal volume of sterile saline (0.85% NaCl). Mix thoroughly. To each 500 ml of melted liver veal agar at 50°C, add 40 ml of the egg yolk-saline suspension. Mix thoroughly and pour plates. Dry plates at room temperature for 2 days or at 35°C for 24 hours. Discard contaminated plates and store sterile plates in the refrigerator.

40.23 Malonate Broth

Yeast extract	1 g
Ammonium sulfate	2 g
Dipotassium phosphate	0.6 g
Monopotassium phosphate	0.4 g
Sodium chloride	2 g
Sodium malonate	3 g
Glucose	0.25 g
Bromthymol blue	0.025 g
Distilled H_2O	1 L

Dissolve by heating if necessary. Dispense 3 ml portions into 13 x 100 mm test tubes and autoclave for 15 minutes at 121°C. Final pH 6.7 $\pm$ 0.1.

40.24 **Malt Extract Broth**

Malt extract	15 g
Distilled H_2O	1 L

Adjust pH to 4.7. Dispense into tubes and autoclave for 15 minutes at 121°C. Subject medium to as little heat as possible.

40.25 Motility-nitrate Medium

Beef extract	3 g
Bacto peptone	5 g
KNO_3	1 g
Agar	3 g
Distilled H_2O	1000 ml

Heat to dissolve agar. Adjust to pH 7.0, mix thoroughly and dispense 11 ml in 16 x 150 mm tubes. Autoclave 15 minutes at 121°C.

(May use Difco nitrate broth with 3 g of agar added per liter.)

40.26 Motility Test Medium

Beef extract	3 g
Peptone	10 g
NaCl	5 g
Agar	4 g
Distilled H_2O	1 L

Heat gently to dissolve. Boil 1-2 minutes. Dispense 20 ml into test tubes and autoclave for 15 minutes at 121°C. Final pH 7.4 ± 0.1.

40.27 Nonfat Dry Milk Solution (reconstituted)

Add 1000 ml of distilled water to 100 g nonfat dry milk. Mix well. Autoclave 20 minutes at 121°C. Cool before using.

40.28 Nutrient Agar

To nutrient broth formula (40.29), add 15 g of agar per liter.

40.29 Nutrient Broth

Suspend 3 g of beef extract, 5 g of peptone (DIFCO) or 5 g of Gelysate, and 3 g of beef extract (BBL) in 1 L of distilled water. Heat with occasional agitation until ingredients dissolve. Autoclave 15 minutes at 121°C. Cool before using.

40.30 Penassay Seed Agar

Peptone	6.0 g
Pancreatic digest of casein	4.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Dextrose	1.0 g
Agar	15.0 g
Distilled H_2O	1.0 L

Boil to dissolve. Dispense in 100 ml portions. Final pH 6.5-6.6.

40.31 Selenite-Cystine Broth

Tryptone	5 g
Lactose	4 g
Na_2HPO_4	10 g
Sodium selenite	4 g
Cystine	0.01 g
Distilled H_2O	1 L

Dissolve and heat with frequent agitation. Dispense 10 ml portions into sterile 16 x 150 mm test tubes. Do not autoclave. Heat 10 minutes in flowing steam. Final pH 7.0 ± 0.1. Medium is not sterile. Use same day as prepared.

40.32 Sulfite-Polymyxin-Sulfadiazine (SPS) Agar

Tryptone	15 g
Yeast extract	10 g
Ferric citrate	0.5 g
Agar	15 g
Distilled H_2O	1000 ml

Adjust to pH 7. Autoclave for 15 minutes at 121°C. To each liter of sterile medium add the following amounts of filter-sterilized solutions:

5 ml of 10% sodium sulfite ($Na_2SO_3.7H_2O$) solution

10 ml of 0.1% polymyxin B sulfate solution

10 ml of sodium sulfadiazine solution containing 12 mg/ml.

40.33 Triple Sugar Iron Agar (TSI)

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	15.0 g
Proteose peptone	5.0 g
Lactose	10.0 g
Saccharose	10.0 g
Dextrose	1.0 g
Ferrous sulfate	0.2 g
Sodium chloride	5.0 g
Sodium thiosulfate	0.3 g
Agar	12.0 g
Phenol red	0.024 g
Distilled H_2O	1.0 L

Suspend ingredients in 1 L of H_2O, mix thoroughly, and heat with occasional agitation. Boil ca 1 minute until ingredients dissolve. Fill tubes and autoclave 12 minutes at 121°C. Before media solidifies, slant tubes so that deep butts and adequate slants are formed. Final pH 7.3-7.4 $\pm$ 0.1.

40.34 Trypticase Peptone Glucose Yeast Extract Broth with Sterile Trypsin Added (TPGYT)*

(a) Trypticase peptone glucose yeast extract broth

Trypticase	50 g
Bacto-peptone	5 g
Yeast extract	20 g
Dextrose	4 g
Sodium thioglycollate	1 g
Distilled H_2O	1000 ml

Adjust to pH 7.0. Dispense the medium in volumes appropriate for the use: 15 ml in 20 mm culture tubes or 100 ml in 6 oz. prescription bottles. Autoclave the dispensed medium at 121°C for 6 minutes and 12 minutes, respectively. Refrigerate and discard if not used within 2 weeks.

(b) Prepare a 1.5% aqueous solution of trypsin (Difco 1:250). Sterilize by filtration through a Millipore filter. The trypsin solution should be prepared just before it is added to the TPGY medium.

(c) Boil the TPGY broth for 10-15 minutes (depending on the volume) in an Arnold steamer or in an autoclave set for flowing steam. Cool immediately after steaming. After cooling, add an appropriate amount of the sterilized trypsin solution (b) to the TPGY medium (a) to give a 0.1% final concentration. Mix very carefully, avoiding the addition of air to the medium as much as possible. After the addition of trypsin to the TPGY broth, the medium cannot be stored.

40.35 Trypticase or Tryptic Soy Agar

Trypticase or tryptone	15.0 g
Phytone or soytone	5.0 g
NaCl	5.0 g
Agar	15.0 g

Suspend ingredients in 1 L of H_2O, mixing thoroughly. Heat with frequent agitation, and boil for about 1 minute to dissolve completely. Autoclave 15 minutes at 121°C. Final pH 7.3 $\pm$ 0.1.

40.36 Tryptone-Sulfite-Neomycin (TSN) Agar

Tryptone	15 g
Yeast extract	10 g
Ferric citrate (soluble)	0.5 g
Sodium sulfite	1.0 g
Agar	15 g
Distilled H_2O	1000 ml

Adjust to pH 7.2. Autoclave for 15 minutes at 121°C. To each liter of sterile medium add the following amounts of filter-sterilized solutions:

10 ml of 0.2% polymyxin B sulfate

10 ml of 0.5% neomycin sulfate

Final concentration in the medium: 20 µg/ml polymyxin, 50 µg/ml neomycin.

40.37 Urea Broth (DIFCO); Urease Test Medium (BBL)

Yeast extract	0.1 g
Monopotassium phosphate	9.1 g
Disodium phosphate	9.5 g
Urea	20.0 g
Phenol red	0.01 g
Distilled H_2O	1.0 L

Dissolve ingredients. Filter-sterilize and distribute aseptically 1.5-3 ml portions into 13 x 100 mm test tubes. Do not heat. Final pH 6.8 $\pm$ 0.1.

40.38 Vogel-Johnson (V-J) Agar (Tellurite Glycine Red Agar Base)

Trypticase or tryptone	10.0 g
Yeast extract	5.0 g
d-Mannitol	10.0 g
K_2HPO_4	5.0 g
$LiCl.6H_2O$	5.0 g
Glycine	10.0 g
Agar	15.0 g
Phenol red	0.025 g

Suspend ingredients in 1 L of H_2O and mix thoroughly. Heat with frequent agitation and boil 1 minute. Dispense in 100 ml portions and autoclave 15 minutes at 121°C. Cool to 45-50°C. To each 100 ml, add 2 ml of 1% K tellurite solution, which has been autoclaved separately 15 minutes at 121°C. Mix gently. Final pH, 7.2 $\pm$ 0.2. Pour 15-18 ml into sterile 100 x 15 mm petri dishes. Temper plates at room temperature for ca 48 hours to reduce surface moisture.

40.39 Xylose Lysine Deoxycholate Agar (XLD)

Yeast extract	3 g
L-Lysine	5 g
Xylose	3.75 g
Lactose	7.5 g
Sucrose	7.5 g
Na deoxycholate	2.5 g
Ferric ammonium citrate	0.8 g
Na thiosulfate	6.8 g
NaCl	5 g
Agar	15 g
Phenol red	0.08 g
Distilled H_2O	1 L

Boil to dissolve completely. Do not overheat. Pour into plates as soon as medium has cooled. Overheating may cause precipitation but reactions will be satisfactory although colonies may be slightly smaller. Final pH 7.4.

40.40 Lauryl Sulfate Tryptose Broth

Trypticase or tryptose	20.0 g
NaCl	5.0 g
Lactose	5.0 g
K_2HPO_4	2.75 g
KH_2PO_4	2.75 g
Sodium lauryl sulfate	0.1 g
Distilled H_2O	1 L

Dispense into test tubes containing inverted fermentation tubes (10 x 75 mm) and autoclave at 121°C for 15 minutes. Final pH 6.8 ± 0.1.

40.41 Plate Count Agar

Pancreatic digest of casein, USP	5.0 g
Yeast extract	2.5 g
Glucose	1.0 g
Agar	15.0 g
Distilled H_2O	1.0 L

Suspend ingredients in water. Heat to boiling until all ingredients are dissolved. Autoclave at 121°C for 15 minutes. Final pH 7.0 ± 0.1.

41. REAGENTS, DILUENTS, ETC.

alpha-Naphthol solution.....41.01
Brilliantgreen solution (1% aqueous).....41.02
Butterfield's buffered phosphate diluent.....41.03
Crystal violet solution (1% aqueous).....41.04
Desiccated coagulase plasma (rabbit) with EDTA.....41.05
Gel-phosphate diluent.....41.06
Kovac's reagent.....41.07
Methylene blue stain (Loeffler's).....41.08
Methyl red solution.....41.09
Saline (0.85% solution).....41.10
Peptone water diluent.....41.11
Reagent I.....41.12
Reagent II.....41.13
Tergitol Anionic 7 (sodium heptadecyl sulfate).....41.14
Voges-Proskauer reagent (V-P).....41.15

41.01 alpha-Naphthol Solution

alpha-Naphthol	5 g
Absolute ethyl alcohol	100 ml

41.02 Brilliant Green Solution (1% aqueous)

Dissolve 1 g of brilliant green dye in sterile distilled water and dilute to 100 ml.

41.03 Butterfield's Buffered Phosphate Diluent

(a) Stock solution. Dissolve 34 g of KH_2PO_4 in 500 ml of distilled water, adjust to pH 7.2 with ca 175 ml of 1 N NaOH, and dilute to 1 L. Store under refrigeration.

(b) Diluent. Dilute 1.25 ml of stock solution (a) to 1 L with distilled water. Prepare dilution blanks using this solution, dispensing a sufficient quantity to allow for losses due to autoclaving.

Autoclave for 15 minutes at 121°C.

41.04 Crystal Violet Solution (1% aqueous)

Dissolve 1 g of crystal violet dye in sterile distilled water and dilute to 100 ml.

41.05 Desiccated Coagulase Plasma (Rabbit) with EDTA

Reconstitute according to directions of manufacturer. If not available, reconstitute "Desiccated coagulase plasma (rabbit)" and add disodium EDTA to make final concentration of 0.1%.

41.06 Gel-Phosphate Diluent

Gelatin	2 g
Na_2HPO_4	4 g
Distilled H_2O	1 L

Adjust to pH 6.2 with 1 N HCl. Autoclave for 20 minutes at 121°C.

41.07 Kovac's Reagent

p-Dimethylaminobenzaldehyde	5 g
Amyl alcohol	75 ml
Concentrated HCl	25 ml

Dissolve the p-dimethylaminobenzaldehyde in the amyl alcohol; then add the HCl. Test the reagent on a known sample of indole to obtain the typical red color before using it on unknowns.

41.08 Methylene Blue Stain (Loeffler's)

Solution A

Methylene blue (90% dye content)	0.3 g
Ethyl alcohol (95%)	30 ml

Solution B

Dilute KOH (0.01%)	100 ml

Mix solutions A and B.

41.09 Methyl Red Solution

Methyl red	0.1 g
95% ethyl alcohol	300 ml
Distilled H_2O	200 ml

Dissolve the methyl red in the alcohol. Add the water.

41.10 0.85% NaCl

Sodium chloride	8.5 g
Distilled H_2O	1 L

41.11 Peptone Water Diluent

Bacto peptone	1 g
Distilled H_2O	1000 ml

Adjust to pH 6.8. Prepare dilution blanks with this solution, dispensing a sufficient quantity to allow for loss during autoclaving. Autoclave at 121°C for 15 minutes.

41.12 **Reagent I**

Sulfanilic acid	0.8 g
5 N acetic acid[1/]	100.0 ml

41.13 **Reagent II**

alpha-Naphthylamine or dimethyl-alpha-naphthylamine	0.6 ml
5 N acetic acid[1/]	100.0 ml

41.14 **Tergitol Anionic 7 (Sodium Heptadecyl Sulfate)**

Steam for 30 minutes. Cool before using.

41.15 **Voges-Proskauer (V-P) Reagent**

KOH	40 g
Distilled H_2O	100 ml

[1/] 28.75 ml of glacial acetic acid added to 71.25 ml of distilled water

MPN Tables

42.01 MPN per gram of sample planting 1.0, 0.1, and 0.01 ml portions.

No. of Positive Tubes			MPN Per Gram	No. of Positive Tubes			MPN Per Gram
1.0 g	0.1 g	0.01 g		1.0 g	0.1 g	0.01 g	
0	0	0	0.0	2	0	0	0.91
0	0	1	0.3	2	0	1	1.4
0	0	2	0.6	2	0	2	2.0
0	0	3	0.9	2	0	3	2.6
0	1	0	0.3	2	1	0	1.5
0	1	1	0.61	2	1	1	2.0
0	1	2	0.92	2	1	2	2.7
0	1	3	1.2	2	1	3	3.4
0	2	0	0.62	2	2	0	2.1
0	2	1	0.93	2	2	1	2.8
0	2	2	1.2	2	2	2	3.5
0	2	3	1.6	2	2	3	4.2
0	3	0	0.94	2	3	0	2.9
0	3	1	1.3	2	3	1	3.6
0	3	2	1.6	2	3	2	4.4
0	3	3	1.9	2	3	3	5.3
1	0	0	0.36	3	0	0	2.3
1	0	1	0.72	3	0	1	3.9
1	0	2	1.1	3	0	2	6.4
1	0	3	1.5	3	0	3	9.5
1	1	0	0.73	3	1	0	4.3
1	1	1	1.1	3	1	1	7.5
1	1	2	1.5	3	1	2	12.0
1	1	3	1.9	3	1	3	16.0
1	2	0	1.1	3	2	0	9.3
1	2	1	1.5	3	2	1	15.0
1	2	2	2.0	3	2	2	21.0
1	2	3	2.4	3	2	3	29.0
1	3	0	1.6	3	3	0	24.0
1	3	1	2.0	3	3	1	46.0
1	3	2	2.4	3	3	2	110.0
1	3	3	2.9	3	3	3	>110.0

MPN Tables, Cont.

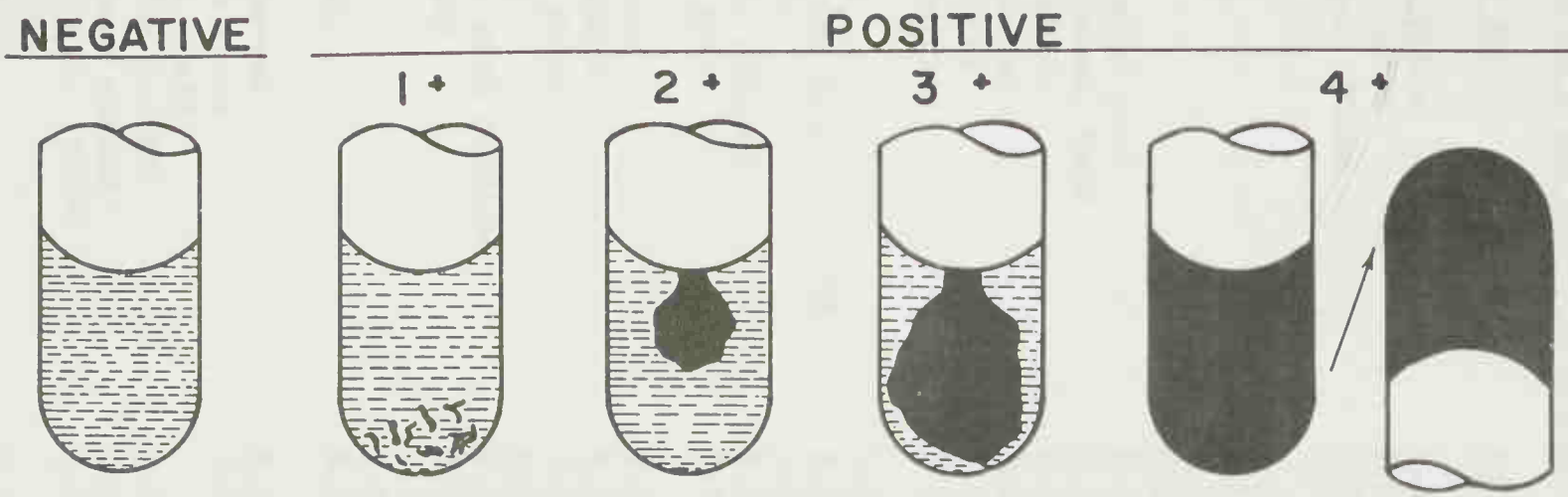
MPN per gram of sample planting 0.1, 0.01 and 0.001 ml portions

No. of Positive Tubes			MPN Per Gram	No. of Positive Tubes			MPN Per Gram
0.1 g	0.01 g	0.001 g		0.1 g	0.01 g	0.001 g	
0	0	0	0.0	2	0	0	9.1
0	0	1	3.0	2	0	1	14.0
0	0	2	6.0	2	0	2	20.0
0	0	3	9.0	2	0	3	26.0
0	1	0	3.0	2	1	0	15.0
0	1	1	6.1	2	1	1	20.0
0	1	2	9.2	2	1	2	27.0
0	1	3	12.0	2	1	3	34.0
0	2	0	6.2	2	2	0	21.0
0	2	1	9.3	2	2	1	28.0
0	2	2	12.0	2	2	2	35.0
0	2	3	16.0	2	2	3	42.0
0	3	0	9.4	2	3	0	29.0
0	3	1	13.0	2	3	1	36.0
0	3	2	16.0	2	3	2	44.0
0	3	3	19.0	2	3	3	53.0
1	0	0	3.6	3	0	0	23.0
1	0	1	7.2	3	0	1	39.0
1	0	2	11.0	3	0	2	64.0
1	0	3	15.0	3	0	3	95.0
1	1	0	7.3	3	1	0	43.0
1	1	1	11.0	3	1	1	75.0
1	1	2	15.0	3	1	2	120.0
1	1	3	19.0	3	1	3	160.0
1	2	0	11.0	3	2	0	93.0
1	2	1	15.0	3	2	1	150.0
1	2	2	20.0	3	2	2	210.0
1	2	3	24.0	3	2	3	290.0
1	3	0	16.0	3	3	0	240.0
1	3	1	20.0	3	3	1	460.0
1	3	2	24.0	3	3	2	1,100.0
1	3	3	29.0	3	3	3	>1,100.0

"The clot produced varies from one which is solid and immovable when the tube is inverted, to a loose clot or occasionally only a trace of fibrin. Any degree of clotting is regarded as a positive reaction."

—*Blair, J.E. in Dubos' "Bocterial and Mycotic Infections of Mon," Lippincott, 1952, p. 372.*

42.02 TYPES OF COAGULASE TEST REACTIONS



NEGATIVE	NO EVIDENCE OF FIBRIN FORMATION
1+ POSITIVE	SMALL UNORGANIZED CLOTS
2+ POSITIVE	SMALL ORGANIZED CLOT
3+ POSITIVE	LARGE ORGANIZED CLOT
4+ POSITIVE	ENTIRE CONTENT OF TUBE COAGULATES AND IS NOT DISPLACED WHEN TUBE IS INVERTED

—From Turner, F.J. & Schwortz, B.S. The Use of o Lyophilized Humon Plasmo, Standordized for Blood Clotting Factors, in the Coagulose Test. Presented before the Society of Americon Bacteriologists, Houston, May 3, 1956

42.03 pH Range of a Few Selected Commercially Canned Foods

Kind of Food	pH Range, approximate
Apples, whole	3.4 - 3.5
Apples, juice	3.3 - 3.5
Asparagus, green	5.0 - 5.8
Beans	
baked	4.8 - 5.5
green	4.9 - 5.5
lima	5.4 - 6.3
soy	6.0 - 6.6
Beans, with pork	5.1 - 5.8
Beef, corned, hash	5.5 - 6.0
Beets, whole	4.9 - 5.8
Blackberries	3.0 - 4.2
Blueberries	3.2 - 3.6
Boysenberries	3.0 - 3.3
Bread	
white	5.0 - 6.0
date and nut	5.1 - 5.6
Broccoli	5.2 - 6.0
Carrots, chopped	5.3 - 5.6
Carrot juice	5.2 - 5.8
Cheese	
Parmesan	5.2 - 5.3
Roquefort	4.7 - 4.8
Cherry juice	3.4 - 3.6
Chicken	6.2 - 6.4
Chicken with noodles	6.2 - 6.7
Chop suey	5.4 - 5.6
Cider	2.9 - 3.3
Clams	5.9 - 7.1
Cod fish	6.0 - 6.1
Corn	
on-the-cob	6.1 - 6.8
cream style	5.9 - 6.5
whole grain	
brine packed	5.8 - 6.5
vacuum packed	6.0 - 6.4
Crab apples, spiced	3.3 - 3.7
Cranberry	
juice	2.5 - 2.7
sauce	2.3 - 2.3
Currant juice	3.0 - 3.0

Kind of Food	pH Range, approximate
Dates	6.2 - 6.4
Duck	6.0 - 6.1
Figs	4.9 - 5.0
Frankfurters	6.2 - 6.2
Fruit cocktail	3.6 - 4.0
Gooseberries	2.8 - 3.1
Grapefruit	
juice	2.9 - 3.4
pulp	3.4 - 3.4
sections	3.0 - 3.5
Grapes	3.5 - 4.5
Ham, spiced	6.0 - 6.3
Hominy, lye	6.9 - 7.9
Huckleberries	2.8 - 2.9
Jam, fruit	3.5 - 4.0
Jellies, fruit	3.0 - 3.5
Lemons	2.2 - 2.4
juice	2.2 - 2.6
Lime juice	2.2 - 2.4
Loganberries	2.7 - 3.5
Mackerel	5.9 - 6.2
Milk	
cow	6.4 - 6.8
evaporated	5.9 - 6.3
Molasses	5.0 - 5.4
Mushrooms	6.0 - 6.5
Olives, ripe	5.9 - 7.3
Orange juice	3.0 - 4.0
Oysters	6.3 - 6.7
Peaches	3.4 - 4.2
Pears (Bartlett)	3.8 - 4.6
Peas	5.6 - 6.5
Pickles	
dill	2.6 - 3.8
sour	3.0 - 3.5
sweet	2.5 - 3.0
Pimento	4.3 - 4.9
Pineapple	
crushed	3.2 - 4.0
sliced	3.5 - 4.1
juice	3.4 - 3.7
Plums	2.8 - 3.0

Kind of Food	pH Range, approximate
Potatoes	
white	5.4 - 5.9
mashed	5.1 - 5.1
Potato salad	3.9 - 4.6
Prune juice	3.7 - 4.3
Pumpkin	5.2 - 5.5
Raspberries	2.9 - 3.7
Rhubarb	2.9 - 3.3
Salmon	6.1 - 6.5
Sardines	5.7 - 6.6
Sauerkraut	3.1 - 3.7
juice	3.3 - 3.4
Shrimp	6.8 - 7.0
Soups	
bean	5.7 - 5.8
beef broth	6.0 - 6.2
chicken noodle	5.5 - 6.5
clam chowder	5.6 - 5.9
duck	5.0 - 5.7
mushroom	6.3 - 6.7
noodle	5.6 - 5.8
oyster	6.5 - 6.9
pea	5.7 - 6.2
tomato	4.2 - 5.2
turtle	5.2 - 5.3
vegetable	4.7 - 5.6
Spinach	4.8 - 5.8
Squash	5.0 - 5.3
Strawberries	3.0 - 3.9
Sweet potatoes	5.3 - 5.6
Tomatoes	4.1 - 4.4
juice	3.9 - 4.4
Tuna	5.9 - 6.1
Turnip greens	5.4 - 5.6
Vegetable	
juice	3.9 - 4.3
mixed	5.4 - 5.6
Vinegar	2.4 - 3.4
Youngberries	3.0 - 3.7

Kind of Food	pH Range, approximate
Other miscellaneous products:	
Beers	4.0 - 5.0
Ginger ale	2.0 - 4.0
Human	
blood plasma	7.3 - 7.5
duodenal content	4.8 - 8.2
feces	4.6 - 8.4
gastric contents	1.0 - 3.0
milk	6.6 - 7.6
saliva	6.0 - 7.6
spinal fluid	7.3 - 7.5
urine	4.8 - 8.4
Magnesia, milk of	10.0 - 10.5
Water	
distilled, CO_2	6.8 - 7.0
mineral	6.2 - 9.4
sea	8.0 - 8.4
Wines	2.3 - 3.8

42.04 List of Official Methods

(a) Frozen and/or Prepared Foods, and Tree Nut Meats. Determination of aerobic plate count, coliform group, Escherichia coli and coagulase-positive staphylococci. JAOAC 49 (1), 246-250 (1966); and revisions, JAOAC 51 (2), 505-506 (1968).

(b) Detection and Identification of Salmonella in Dried Whole Eggs, Dried Egg Yolk, and Dried Egg White. JAOAC 50 (1), 231-239 (1967); and revisions JAOAC 51 (2), 505-506 (1968).

(c) Examination of Eggs and Egg Products. Determination of aerobic plate count and direct microscopic count. AOAC (10th edition, 1965) 37.003-37.006, 37.010; and latest revisions.

(d) Shellfish (clams, oysters, mussels, etc.). "Recommended Methods for the Bacteriological Examination of Sea Water and Shellfish." APHA, 1962.

(e) Water and Beverages. Latest edition of "Standard Methods for the Examination of Water and Wastewater." APHA

42.05 List of Other Recommended Methods

(a) Penicillin Contamination in Drugs. "Procedure for Detecting and Measuring Penicillin Contamination in Drugs." Department of Health, Education, and Welfare, Food and Drug Administration, Bureau of Scientific Standards and Evaluation, Division of Antibiotics and Insulin Certification, Washington, D. C. 20204 (1965); addendum August 1967.

(b) Antibiotic Residues in Milk, Dairy Products and Animal Tissue. "Antibiotic Residues in Milk, Dairy Products, and Animal Tissues: Methods, Reports, and Protocols" (Revised October 1968). National Center for Antibiotic and Insulin Analysis, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C. 20204.

42.06 FOUR-PLACE LOGARITHMS

N	0	1	2	3	4	5	6	7	8	9	Proportional Parts 1	2	3	4	5	6	7	8	9
10	0000	0043	0086	0128	0170	0212	0253	0294	0334	0374	*4	8	12	17	21	25	29	33	37
11	0414	0453	0492	0531	0569	0607	0645	0682	0719	0755	4	8	11	15	19	23	26	30	34
12	0792	0828	0864	0899	0934	0969	1004	1038	1072	1106	3	7	10	14	17	21	24	28	31
13	1139	1173	1206	1239	1271	1303	1335	1367	1399	1430	3	6	10	13	16	19	23	26	29
14	1461	1492	1523	1553	1584	1614	1644	1673	1703	1732	3	6	9	12	15	18	21	24	27
15	1761	1790	1818	1847	1875	1903	1931	1959	1987	2014	*3	6	8	11	14	17	20	22	25
16	2041	2068	2095	2122	2148	2175	2201	2227	2253	2279	3	5	8	11	13	16	18	21	24
17	2304	2330	2355	2380	2405	2430	2455	2480	2504	2529	2	5	7	10	12	15	17	20	22
18	2553	2577	2601	2625	2648	2672	2695	2718	2742	2765	2	5	7	9	12	14	16	19	21
19	2788	2810	2833	2856	2878	2900	2923	2945	2967	2989	2	4	7	9	11	13	16	18	20
20	3010	3032	3054	3075	3096	3118	3139	3160	3181	3201	2	4	6	8	11	13	15	17	19
21	3222	3243	3263	3284	3304	3324	3345	3365	3385	3404	2	4	6	8	10	12	14	16	18
22	3424	3444	3464	3483	3502	3522	3541	3560	3579	3598	2	4	6	8	10	12	14	15	17
23	3617	3636	3655	3674	3692	3711	3729	3747	3766	3784	2	4	6	7	9	11	13	15	17
24	3802	3820	3838	3856	3874	3892	3909	3927	3945	3962	2	4	5	7	9	11	12	14	16
25	3979	3997	4014	4031	4048	4065	4082	4099	4116	4133	2	3	5	7	9	10	12	14	15
26	4150	4166	4183	4200	4216	4232	4249	4265	4281	4298	2	3	5	7	8	10	11	13	15
27	4314	4330	4346	4362	4378	4393	4409	4425	4440	4456	2	3	5	6	8	9	11	13	14
28	4472	4487	4502	4518	4533	4548	4564	4579	4594	4609	2	3	5	6	8	9	11	12	14
29	4624	4639	4654	4669	4683	4698	4713	4728	4742	4757	1	3	4	6	7	9	10	12	13
30	4771	4786	4800	4814	4829	4843	4857	4871	4886	4900	1	3	1	6	7	9	10	11	13
31	4914	4928	4942	4955	4969	4983	4997	5011	5024	5038	1	3	4	6	7	8	10	11	12
32	5051	5065	5079	5092	5105	5119	5132	5145	5159	5172	1	3	4	5	7	8	9	11	12
33	5185	5198	5211	5224	5237	5250	5263	5276	5289	5302	1	3	4	5	6	8	9	10	12
34	5315	5328	5340	5353	5366	5378	5391	5403	5416	5428	1	3	4	5	6	8	9	10	11
35	5441	5453	5465	5478	5490	5502	5514	5527	5539	5551	1	2	4	5	6	7	9	10	11
36	5563	5575	5587	5599	5611	5623	5635	5647	5658	5670	1	2	4	5	6	7	8	10	11
37	5682	5694	5705	5717	5729	5740	5752	5763	5775	5786	1	2	3	5	6	7	8	9	10
38	5798	5809	5821	5832	5843	5855	5866	5877	5888	5899	1	2	3	5	6	7	8	9	10
39	5911	5922	5933	5944	5955	5966	5977	5988	5999	6010	1	2	3	4	5	7	8	9	10
40	6021	6031	6042	6053	6064	6075	6085	6096	6107	6117	1	2	3	4	5	6	8	9	10
41	6128	6138	6149	6160	6170	6180	6191	6201	6212	6222	1	2	3	4	5	6	7	8	9
42	6232	6243	6253	6263	6274	6284	6294	6304	6314	6325	1	2	3	4	5	6	7	8	9
43	6335	6345	6355	6365	6375	6385	6395	6405	6415	6425	1	2	3	4	5	6	7	8	9
44	6435	6444	6454	6464	6474	6484	6493	6503	6513	6522	1	2	3	4	5	6	7	8	9
45	6532	6542	6551	6561	6571	6580	6590	6599	6609	6618	1	2	3	4	5	6	7	8	9
46	6628	6637	6646	6656	6665	6675	6684	6693	6702	6712	1	2	3	4	5	6	7	7	8
47	6721	6730	6739	6749	6758	6767	6776	6785	6794	6803	1	2	3	4	5	5	6	7	8
48	6812	6821	6830	6839	6848	6857	6866	6875	6884	6893	1	2	3	4	4	5	6	7	8
49	6902	6911	6920	6928	6937	6946	6955	6964	6972	6981	1	2	3	4	4	5	6	7	8
50	6990	6998	7007	7016	7024	7033	7042	7050	7059	7067	1	2	3	3	4	5	6	7	8
51	7076	7084	7093	7101	7110	7118	7126	7135	7143	7152	1	2	3	3	4	5	6	7	8
52	7160	7168	7177	7185	7193	7202	7210	7218	7226	7235	1	2	2	3	4	5	6	7	7
53	7243	7251	7259	7267	7275	7284	7292	7300	7308	7316	1	2	2	3	4	5	6	6	7
54	7324	7332	7340	7348	7356	7364	7372	7380	7388	7396	1	2	2	3	4	5	6	6	7
N	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9

* Interpolation in this section of the table is inaccurate.

FOUR-PLACE LOGARITHMS (Continued)

N	0	1	2	3	4	5	6	7	8	9	Proportional Parts 1	2	3	4	5	6	7	8	9
55	7404	7412	7419	7427	7435	7443	7451	7459	7466	7474	1	2	2	3	4	5	5	6	7
56	7482	7490	7497	7505	7513	7520	7528	7536	7543	7551	1	2	2	3	4	5	5	6	7
57	7559	7566	7574	7582	7589	7597	7604	7612	7619	7627	1	2	2	3	4	5	5	6	7
58	7634	7642	7649	7657	7664	7672	7679	7686	7694	7701	1	1	2	3	4	4	5	6	7
59	7709	7716	7723	7731	7738	7745	7752	7760	7767	7774	1	1	2	3	4	4	5	6	7
60	7782	7789	7796	7803	7810	7818	7825	7832	7839	7846	1	1	2	3	4	4	5	6	6
61	7853	7860	7868	7875	7882	7889	7896	7903	7910	7917	1	1	2	3	4	4	5	6	6
62	7924	7931	7938	7945	7952	7959	7966	7973	7980	7987	1	1	2	3	3	4	5	6	6
63	7993	8000	8007	8014	8021	8028	8035	8041	8048	8055	1	1	2	3	3	4	5	5	6
64	8062	8069	8075	8082	8089	8096	8102	8109	8116	8122	1	1	2	3	3	4	5	5	6
65	8129	8136	8142	8149	8156	8162	8169	8176	8182	8189	1	1	2	3	3	4	5	5	6
66	8195	8202	8209	8215	8222	8228	8235	8241	8248	8254	1	1	2	3	3	4	5	5	6
67	8261	8267	8274	8280	8287	8293	8299	8306	8312	8319	1	1	2	3	3	4	5	5	6
68	8325	8331	8338	8344	8351	8357	8363	8370	8376	8382	1	1	2	3	3	4	4	5	6
69	8388	8395	8401	8407	8414	8420	8426	8432	8439	8445	1	1	2	2	3	4	4	5	6
70	8451	8457	8463	8470	8476	8482	8488	8494	8500	8506	1	1	2	2	3	4	4	5	6
71	8513	8519	8525	8531	8537	8543	8549	8555	8561	8567	1	1	2	2	3	4	4	5	5
72	8573	8579	8585	8591	8597	8603	8609	8615	8621	8627	1	1	2	2	3	4	4	5	5
73	8633	8639	8645	8651	8657	8663	8669	8675	8681	8686	1	1	2	2	3	4	4	5	5
74	8692	8698	8704	8710	8716	8722	8727	8733	8739	8745	1	1	2	2	3	4	4	5	5
75	8751	8756	8762	8768	8774	8779	8785	8791	8797	8802	1	1	2	2	3	3	4	5	5
76	8808	8814	8820	8825	8831	8837	8842	8848	8854	8859	1	1	2	2	3	3	4	5	5
77	8865	8871	8876	8882	8887	8893	8899	8904	8910	8915	1	1	2	2	3	3	4	4	5
78	8921	8927	8932	8938	8943	8949	8954	8960	8965	8971	1	1	2	2	3	3	4	4	5
79	8976	8982	8987	8993	8998	9004	9009	9015	9020	9025	1	1	2	2	3	3	4	4	5
80	9031	9036	9042	9047	9053	9058	9063	9069	9074	9079	1	1	2	2	3	3	4	4	5
81	9085	9090	9096	9101	9106	9112	9117	9122	9128	9133	1	1	2	2	3	3	4	4	5
82	9138	9143	9149	9154	9159	9165	9170	9175	9180	9186	1	1	2	2	3	3	4	4	5
83	9191	9196	9201	9206	9212	9217	9222	9227	9232	9238	1	1	2	2	3	3	4	4	5
84	9243	9248	9253	9258	9263	9269	9274	9279	9284	9289	1	1	2	2	3	3	4	4	5
85	9294	9299	9304	9309	9315	9320	9325	9330	9335	9340	1	1	2	2	3	3	4	4	5
86	9345	9350	9355	9360	9365	9370	9375	9380	9385	9390	1	1	2	2	3	3	4	4	5
87	9395	9400	9405	9410	9415	9420	9425	9430	9435	9440	0	1	1	2	2	3	3	4	4
88	9445	9450	9455	9460	9465	9469	9474	9479	9484	9489	0	1	1	2	2	3	3	4	4
89	9494	9499	9504	9509	9513	9518	9523	9528	9533	9538	0	1	1	2	2	3	3	4	4
90	9542	9547	9552	9557	9562	9566	9571	9576	9581	9586	0	1	1	2	2	3	3	4	4
91	9590	9595	9600	9605	9609	9614	9619	9624	9628	9633	0	1	1	2	2	3	3	4	4
92	9638	9643	9647	9652	9657	9661	9666	9671	9675	9680	0	1	1	2	2	3	3	4	4
93	9685	9689	9694	9699	9703	9708	9713	9717	9722	9727	0	1	1	2	2	3	3	4	4
94	9731	9736	9741	9745	9750	9754	9759	9763	9768	9773	0	1	1	2	2	3	3	4	4
95	9777	9782	9786	9791	9795	9800	9805	9809	9814	9818	0	1	1	2	2	3	3	4	4
96	9823	9827	9832	9836	9841	9845	9850	9854	9859	9863	0	1	1	2	2	3	3	4	4
97	9868	9872	9877	9881	9886	9890	9894	9899	9903	9908	0	1	1	2	2	3	3	4	4
98	9912	9917	9921	9926	9930	9934	9939	9943	9948	9952	0	1	1	2	2	3	3	4	4
99	9956	9961	9965	9969	9974	9978	9983	9987	9991	9996	0	1	1	2	2	3	3	3	4
N	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9

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